PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Matthew During *et al.*
Application No:	09/863,179
Filing Date:	May 23, 2001
Entitled:	Glutamic Acid Decarboxylase (GAD) Based Delivery Systems
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Examiner: A.M. Falk

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RULE 132 DECLARATION OF DR. MICHAEL KAPLITT

I, Michael G. Kaplitt, residing at 515 East 72nd Street, Apt. 34D, New York, New York, hereby declare as follows:

1. I received a Bachelor of Arts degree from Princeton University in Molecular Biology in 1987, a Tri-Institutional MD-PhD degree from the The Rockefeller University and Cornell University Medical College in 1993 and 1995. During my postdoctoral training at the Rockefeller University in the Laboratory of Biochemical Genetics and Metabolism, I was also an

intern in surgery and a resident in neurosurgery from 1995-1999. I was a Chief Resident in Neurosurgery at the New York Hospital–Cornell University Medical College from 1999-2000. I was also a Clinical Fellow in the Department of Stereotactic and Functional Neurosurgery at the University of Toronto from 2000-2001. I was appointed Director of the Laboratory of Molecular Neurosurgery, The New York Hospital-Cornell University Medical College and Fellow, The Rockefeller University from 1995-2000. I am currently Assistant Professor of Neurosurgery and Director, Laboratory of Molecular Neurosurgery, Weill Medical College of Cornell University, NY, NY; a Clinical Assistant Attending, Division of Neurosurgery, Dept. of Surgery, Memorial-Sloan Kettering Cancer Center, NY, NY; and an Adjunct Assistant Professor, Laboratory of Neurobiology and Behavior, The Rockefeller University, NY, NY. A copy of my Curriculum Vitae more fully explaining my qualifications, publications and appointments is attached as Exhibit A. I am an inventor on the above-referenced patent application.

2. I am familiar with the patent application at issue and, through this declaration, I present the evidence that was requested and discussed during the interview with Examiner Falk, at the United States Patent and Trademark Office, on October 24, 2003. In particular, evidence relating to the broader application of the invention to different neurological disorders or diseases.

3. The claimed invention relates to methods for altering expression of a glutamic acid decarboxylase (GAD) in a region of the brain . This is accomplished by identifying a target site in the central nervous system that requires modification and delivering a vector that comprises a nucleic acid sequence encoding glutamic acid decarboxylase (GAD) to target site of the central nervous system (e.g., a region of the brain), to alter expression of GAD in the region of the brain.

4. The invention also demonstrates the principal that expression of GAD in a region of the brain alleviates the symptoms of Parkinson's disease rodent and primate *in vivo* models for Parkinson's Disease.

5. One of the embodiments of the invention demonstrates that GAD transduction of neurons in the subthalmic nucleus (STN) increases inhibition in the substantia nigra (SN) and decreases the excitatory effect of STN stimulation on neurons in the SN. The results show that changing the excitatory projection from the STN to the SN into an inhibitory projection, using a

gene therapy approach, alleviates the symptoms of Parkinson's disease (*See* page 59, lines 16-20).

6. The same result was repeated in our extended study, the results of which are published by Luo *et al.* in Science "Subthalmic GAD Gene Therapy in a Parkinson's Disease Rat Model" (2003) 298: 425-429 (Exhibit B). This paper demonstrates that GAD gene transfer into glutamatergic excitatory neurons leads to an inhibitory bias with altered network activity. This phenotypic shift provides strong neuroprotection and demonstrates there is plasticity between excitatory and inhibitory neurotransmission in the mammalian brain that results in a therapeutic effect.

7. The same inventive concept of delivering GAD to a region of the central nervous system, can be applied to any CNS disease in which increasing GABA production is desirable.

8. As further evidence that the invention can be applied to different diseases -- as well as different regions of the CNS -- a post-graduate student working at Neurologix, Inc., (licensee of the present invention) carried out the same method disclosed in the instant application in an animal model of epilepsy. This work, described below, shows that the symptoms of epilepsy can be reduced by delivering GAD to a region of the brain involved in epilepsy, e.g., the hippocampus.

9. The epilepsy experiment involved delivering three AAV vectors, AAV/CBA-hGAD65-1.76-WPRE-BGH ("GAD65"); AAV/CBA-hGAD67-WPRE-BGH ("GAD65"); and AAV-EGFP ("EGFP"), into three experimental groups of animals. A fourth sham group ("SHAM") of animals, was subject to injection with an empty needle as a control for injury related to insertion of a needle into the brain tissue. Two microliters of each of the AAV vector, at a genomic titer of 2×10^{10} genomes/ml, was infused bilaterally into the rat hippocampus using stereotaxic surgery.

10. Four weeks after vector administration, 10mg/kg kainic acid (i.p.) was administered to each animal to induce seizures. The animals were observed for the next 90 minutes for a variety of behavioural characteristics and by electroencephalograms of seizure activity. Figure 1 (Exhibit C) shows the results from an electroencephalogram of seizure activity

in the hippocampus of a rat kainic acid model for epilepsy. The data shows that rats treated with GAD, in particular, GAD65, have reduced seizures compared with the rats treated with EGFP or the SHAM group that received no vector. The results show that expression of GAD in a region of the brain associated with epilepsy provides neuroprotection against seizures.

11. Further evidence that GAD can be delivered to a selected region of the CNS is presented in Boulis *et al.*, "Stereotactic Gene Based Hypothalamic Neuromodulation" (2002) AANS meeting, Chicago (abstract) (Exhibit D).

12. In Boulis *et al.* an AAV-GAD construct, disclosed in the instant application, was used to deliver GAD to the lateral nucleus of the hypothalamus of rats to augment GABA production in the region. The delivery of GAD resulted in altered gene expression and sustained enhancement of GABA production in a deep brain target, which resulted in an alteration of metabolic behavior.

13. Another example of how GAD can be delivered to selected regions of the brain is shown in Jasmin *et al.* in Nature, "Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex" (2003) 424:316-320. (Exhibit E).

14. In Jasmin *et al.*, GABA neurotransmission in the rostral agranular insular cortex (RAIC) of freely moving rats, was altered by locally increasing GABA using two methods: (a) an enzyme inhibitor; and (b) a double-cassette-defective Herpes Simplex Virus (HSV) vector. Use of gene transfer mediated by a viral vector produced lasting analgesia in the rats by enhancing the descending inhibition of spinal nociceptive neurons. This reference further evidences that a variety of vectors may be used to deliver GAD in a targeted manner and alter GABA levels in relevant regions of the CNS.

15. A further example showing that delivering GAD to a region of the central nervous system, may be applied to any relevant disease is evidenced by a recent article by Levanthal *et al.* in Science "GABA and Its Agonists Improve Visual Cortical Function in Senescent Monkeys" (2003) 300:812-815 (Exhibit F).

16. Leventhal *et al.* demonstrated that the alteration of GABA levels, in a region of the visual cortex (V_1) of aged primates resulted in improved acuity -- including improved orientation and direction selectivity, decreased spontaneous activity and an increased ability to signal visual stimuli. This is further evidence that methods of altering GABA levels, such as delivering GAD to the central nervous system, can be used to address a variety of neurodegenerative diseases.

17. Thus one of ordinary skill in the art, would be able to use the application's disclosure, in addition to the knowledge available in the art, to apply the invention to alter expression of glutamic acid decarboxylase (GAD) in a selected region of the brain.

18. In summary, the disclosure in the application, in combination with the knowledge available in the art, would enable one skilled in the art to perform the full scope of the claimed invention without undue experimentation.

19. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 10001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 11/6/03

Michael G. Kaplitt, M.D., Ph.D.

Curriculum Vitae

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PRESENT POSITIONS

Assistant Professor of Neurosurgery and Director, Laboratory of Molecular Neurosurgery, Weill Medical College of Cornell University, NY, NY
Clinical Assistant Attending, Division of Neurosurgery, Dept. of Surgery, Memorial-Sloan Kettering Cancer Center, NY, NY
Adjunct Assistant Professor, Laboratory of Neurobiology and Behavior, The Rockefeller University, NY,NY

PROFESSIONAL EXPERIENCE

Clinical Fellow, Stereotactic and Functional Neurosurgery, Univ. of Toronto (Western Division), Toronto, Ontario, Canada	2000-2001
Chief Resident in Neurosurgery	1999-2000
Resident in Neurosurgery	1996-1999
Intern in Surgery, The New York Hospital-Cornell University Medical College	1995-1996
Post-Doctoral Fellow, Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University	1995-1999

EDUCATION

Tri-Institutional MD-PhD Program

Cornell University Medical College	1993-1995	MD
The Rockefeller University	1989-1993	PhD (Molecular Neurobiology)
University of Rochester School of Medicine (MD-PhD program)	1987-1989	
Princeton University	1983-1987	AB (Molecular Biology) Cert. of Proficiency in Russian Studies

MEDICAL LICENSURE/BOARD CERTIFICATION

Licensed in NY. Board eligible, American Board of Neurological Surgery.

PERSONAL INFORMATION

Birthplace: Brooklyn, N.Y. Birthdate: September 1, 1965
Marital Status: Separated, Children: 1 Son (Jeremy David Kaplitt)

HONORS, AWARDS AND FELLOWSHIPS

Magna Cum Laude, Princeton University
Certificate of Proficiency in Russian Studies, Princeton University
Varsity Letter, Men's Swimming, Princeton University
Founding Editor-in-Chief, Journal of the Univ. of Rochester Medical Center
National Research Service Award Graduate Fellowship
1992 Albert Cass Traveling Fellowship
1994 Saul R. Korey Award for Experimental Neurology, American Academy of Neurology
Sigma Xi Scientific Honor Society
1998 Distinguished Housestaff Award, The New York Hospital-Cornell Medical Center
2000 Fellowship Award, Medical Research Council of Canada
Second place poster, 2001 Congress of Neurological Surgeons
2002 Charles Elsberg Fellowship in Neurological Surgery, New York Academy of Medicine
2002 New Scholar in Aging Research, Ellison Foundation for Medical Research

PROFESSIONAL ACTIVITIES

Member, Neural Disorders Committee, American Society for Gene Therapy
Course Director, Update in Movement Disorder Surgery CME Course, Weill Medical College of Cornell University
Journal Editorial Board Member, Surgical Neurology
Section Editor for Stereotactic and Functional Neurosurgery, Select Reviews in Neurosurgery
Editor, World Society for Stereotactic and Functional Neurosurgery Website
Program Committee, 2001 Quadrennial Meeting, World Society for Stereotactic and Functional Neurosurgery
Program Committee, 2003 Quadrennial Meeting, American Society for Stereotactic and Functional Neurosurgery
Ad Hoc Reviewer, National Cancer Institute
Ad Hoc Reviewer, National Institutes of Neurological Disorders and Stroke
Admissions Committee Member, Weill Medical College of Cornell University

RESEARCH EXPERIENCE

2001-Present Director, Laboratory of Molecular Neurosurgery, Weill Medical College of Cornell University; Adjunct Faculty, The Rockefeller University

Examining effects of deep brain stimulation upon changes in gene expression in brain and mechanisms of such regulation
Developing new method for use of deep brain stimulation to treat chronic drug addiction
Identified new elements which regulate a novel form of circular replication of adeno-associated virus (AAV); further defining mechanisms and consequences of replication and packaging of these circular forms
Studying the effects of anti-oncogene expression on neuronal function and sensitivity to neurodegenerative disorders
Further developing the first clinical protocol for gene therapy for Parkinson's disease to initiate a Phase I clinical trial

2000-2001 **Fellow, Stereotactic and Functional Neurosurgery, The University of Toronto, and Adjunct Faculty, The Rockefeller University**
Used chronic *in vivo* microdialysis to analyze neurochemical changes in the subthalamic nucleus and substantia nigra of patients undergoing subthalamic nucleus deep brain stimulation for Parkinson's disease
Began exploration of gene expression changes in response to deep brain electrical stimulation in rodent models
1995-2000 **Director, Laboratory of Molecular Neurosurgery, The New York Hospital-Cornell University Medical College and Fellow, The Rockefeller University**
°Applications of viral vectors for study and of genes associated with Alzheimer's disease and exploration of potential gene therapy approaches
°Development of novel viral vectors and packaging systems for improved and safer CNS gene delivery.
°Application of viral vectors to study and treatment of Parkinson's disease, epilepsy, Alzheimer's disease and brain tumors.
°Demonstrated the first expression of a foreign gene in mammalian heart using an adeno-associated viral vector.
°Exploration of influence of estrogen upon brain function and disorders including Alzheimer's disease, ischemia and trauma
°Discovered that steroid receptors can act as enzymes to promote proper folding of certain proteins.
1993-1995 **Post-doctoral fellow, Laboratory of Neurobiology and Behavior, The Rockefeller University and Department of Neurosurgery, Yale University School of Medicine.**
°Applications of novel vectors to treatment of mammalian diseases, with particular emphasis upon adeno-associated viral vectors and adenoviral vectors.
°Demonstrated the first expression of a foreign gene in mammalian brain using an adeno-associated viral vector.
°Developed genetic therapy approach for Parkinson's disease, with therapeutic improvement in a rodent model. Primate studies were initiated with promising results.
°Applications of these vector systems to neuro-oncology were explored, with emphasis upon study of anti-oncogenes and genes associated with paraneoplastic syndromes.
1989-1993 **Doctoral Research, Lab. of Neurobiology and Behavior, The Rockefeller Univ.**
°Use of herpes simplex virus (HSV) defective viral vectors as agents for transfer of foreign gene into the adult mammalian brain in vivo.
°Demonstrated the first expression of a foreign gene in rat brain using a defective HSV vector.
°Applied the vector system as a novel approach to study gene regulation, and identified important elements of the rat preproenkephalin promoter using this system.
°Demonstrated that expression of the neuronal protein GAP-43 can induce formation of processes resembling neurites.
°Use of mutant HSV strains as possible therapeutic agents for the treatment of CNS neoplasms.
1987-1989 **Laboratory of Drs. Thomas Broker and Louise Chow, University of Rochester.**
°Analysis of the DNA binding domain of the human papillomavirus E2C protein, and attempted to develop non-radioactive in situ hybridization techniques for use in analysis of cervical cancer specimens.

1985-1987 **Laboratory of Dr. Thomas Shenk, Princeton University.** °Analysis of the basis for tumorigenicity of adenovirus type 9, a unique strain which specifically causes mammary fibroadenomas only in sexually intact female rats.
Summer,1985 **Molecular Diseases Branch, National Heart, Lung and Blood Institute**
° Assisted in the cloning and sequencing of the human apolipoprotein B-100 gene.

Major Invited Lectures (Not including meeting abstracts/presentations from submissions)
Athena Neuroscience Corp., South San Francisco, CA (1992, 1994)
Rudolph Magnus Institute, University of Utrecht, The Netherlands (1992,1993)
Schering-Plough Pharmaceuticals Corp., NJ (1992)
Tel-Aviv University, Tel Aviv, Israel (1992,1994)
Weizmann Institute for Science, Rehovot Israel (1992)
Burke Rehabilitation Center, White Plains, NY (1994)
Cornell Medical College, Dept. of Neurology Grand Rounds, New York, NY (1994, 1998,2001)
Harvard Medical School, Neurogenetics Unit, Boston, MA (1994)
Netherlands Institute for Brain Research, Amsterdam, The Netherlands (1994)
International Conference on Gene Therapy for CNS Disorders, Philadelphia, PA (1995)
Workshop on Basal Ganglia Disorders, UCLA School of Med., Los Angeles, CA (1995)
Rockefeller University, Clinical Research Seminar Series, New York, NY (1995)
Washington University School of Medicine, St. Louis, MO (1995)
International Congress on Endovascular Interventions X, Phoenix, AZ (1996)
Society for Neuroscience Symposium on Gene Transfer (Sponsored by NIMH, NIH) (1996)
NIH, NINDS, Laboratory of Molecular Disorders and Neuroscience, Bethesda, MD (1997)
Albert Einstein College of Medicine, Dept. of Radiation Oncology, Bronx, NY (1999)
Methodist Hospital, Conference on Movement Disorders, Brooklyn, NY (2000)
New York State Neurosurgical Society, Lake George, NY (2000)
2000 Botterell Symposium, University of Toronto, Toronto, Canada (2000)
Grand Rounds, Dept. of Surgery, Memorial-Sloan Kettering Cancer Center, NY, NY (2001)
International Conference on Aging 2001, Mt. Sinai School of Medicine, NY, NY (2001)
Contemporary Management of Movement Disorders, Practical Clinic, AANS (2001)
Contemporary Management of Movement Disorders, Practical Clinic, CNS (2001)
International Conference on Monitoring Molecules in Neuroscience, Dublin, Ireland (2001)
Contemporary Management of Movement Disorders, Practical Clinic, AANS (2002)
Contemporary Management of Movement Disorders, Practical Clinic, CNS (2002)
Plenary lecture, 5th Annual American Society for Gene Therapy Annual Meeting (2002)
University of Rochester Neurosurgery Grand Rounds and Neuroscience Seminar (2002)
Pain Service Seminar Series, Memorial Sloan-Kettering Cancer Center (2003)
Cornell-Salzburg Medical Seminar in Neurosurgery, Faculty (2003)
Program in Biomedical Science Seminar Series, Boston Univ. School of Medicine (2003)
Gene Therapy: The Next 5 Years, American Society for Microbiology (2003)
Contemporary Management of Movement Disorders, Practical Clinic, CNS (2002)
6th Annual American Society for Gene Therapy Annual Meeting (2003)
Update in Neuro-Oncology, Weill Medical College of Cornell University (2003)

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Books and Book Chapters

1. **Kaplitt MG** and Loewy AD, **Editors**. (1995) Viral Vectors: Gene Therapy and Neuroscience Applications. San Diego, CA: Academic Press.

2. Mobbs CV, **Kaplitt MG** and Pfaff DW (1997) "HIP-70/GRP58/Erp61/PLC-a/CPT: A single gene product and member of the protein disulfide isomerase gene family with thiol oxidoreductase activity subject to neuroendocrine regulation", in Prolyl Hydroxylase, Protein Disulfide Isomerase and Other Structurally Related Proteins (Guzman N, ed.) New York: Marcel Dekker, Inc.

3. **Kaplitt MG** and Loewy AD (1998) "Viral Vectors", in Fundamental Neuroscience (Bloom, et. al. eds) San Diego, CA: Academic Press.

4. **Kaplitt MG** and Lozano, AM (2001) Surgical drug delivery for neurodegenerative diseases. Clin Neurosurg 48:127-144.

5. **Kaplitt MG**, Hutchinson W and Lozano, AM (2001) "Target Localization in Movement Disorder Surgery", in Contemporary Clinical Neurology: Surgical Treatment of Parkinson's Disease and Other Movement Disorders (Tarsy, D. et. al., eds.) Totowa, NJ: Humana Press.

6. **Kaplitt MG**, Dostrovsky J, Hutchinson W and Lozano AM "Microelectrode Recording in Functional Neurosurgery" in Neurosurgery (Wilkins, RH and Rengachary, SS, eds.). New York: McGraw-Hill. In press.

7. **Kaplitt MG** and Loewy AD (2002) "Viral Vectors", in Fundamental Neuroscience, 2nd ed. (Bloom, et. al. eds) San Diego, CA: Academic Press.

8. Kaplitt MG, Rezai AR, Lozano A and Tasker R "Deep Brain Stimulation for Chronic Pain" in Youmans Neurological Surgery, 5th Edition (H.R. Winn, ed.). Philadelphia: Elsevier, In press.

9. **Kaplitt MG** and During MJ, **Editors**. Gene Transfer in the Brain: From Basic Science to Human Therapy. San Diego, CA: Academic Press. In preparation.

Publications

1. Mobbs CV, **Kaplitt MG**, Kow L-M, Pfaff DW (1991) PLC-a: A common mediator of the actionof estrogen and other hormones? *Mol. Cell. Endocrinol.* **80**:C187-C191.

2. **Kaplitt MG**, Pfaus JG, Kleopoulos SP, Hanlon BA, Rabkin SD, Pfaff DW (1991) Expression of a functional foreign gene in adult mammalian brain following in vivo transfer via a herpes simplex virus type 1 defective viral vector. *Mol. Cell. Neurosci.* **2**:320-330.

3. **Kaplitt MG**, Rabkin SD, Pfaff DW (1992) Molecular alterations in nerve cells: Direct manipulation and physiologic mediation. *Curr. Top. Neuroendocrinol.* **11**:169-191.

4. **Kaplitt MG**, Kleopoulos SP, Pfaff DW, Mobbs CV. (1993) Estrogen induces HIP-70/PLC-amessenger RNA in the uterus and ventromedial hypothalamus. *Endocrinology* **133**:99-104.

5. Brooks PJ, **Kaplitt MG**, Kleopoulos SP, Funabashi T, Mobbs CV, Pfaff DW. (1993) Sensitive detection of low abundance RNAs by in situ hybridization using single-stranded DNA probes produced by amplified primer extension labeling. *J. Histochem. Cytochem.* **41**:1761-1766.

6. **Kaplitt MG**, Kwong AD, Kleopoulos SP, Mobbs CV, Rabkin SD, Pfaff DW. (1994) Preproenkephalin promoter yields region-specific and long-term expression in adult rat brain following in vivo transfer via a defective herpes simplex viral vector. *Proc. Natl. Acad. Sci. USA*, **91**:8979-8983.

7. **Kaplitt MG**, Leone P, Samulski RJ, Xiao X, Pfaff DW, OMalley K, During MJ (1994) Long-term expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Gen.* **8**:148-154.

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14. Rosenfeld MR, Meneses PM, Dalmau J, Drobnjak M, Cordon-Cardo C, **Kaplitt MG** (1995) Gene transfer of wild-type p53 results in restoration of tumor-suppressor function in a medulloblastoma cell line. *Neurology* 45:1533-1539.

15. Tjuvajev J, Gansbacher B, Desai R, Beattie B, **Kaplitt MG**, Matei C, Koutcher J, Gilboa E, Blasberg R. (1995) RG-2 glioma growth attenuation and severe brain edema caused by local production of interleukin-2 and interferon-gamma. *Cancer Res.* **55**:1902-1910.

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18. **Kaplitt MG**, Xiao X, Samulski RJ, Li R, Ojamaa K, Klein IL, Makimura H, Kaplitt MJ, Strumpf RK, Diethrich EB (1996) Long-term gene transfer in porcine myocardium after coronary infusion of an adeno-associated virus vector. *Ann. Thoracic Surg.* **62**(6): 1669-76.

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28. During MJ, Samulski RJ, Elsworth JD, **Kaplitt MG**, Leone P, Xiao X, Li J, Freese A, Taylor JR, Roth RH, Sladek JR, O'Malley KL, Redmond DE (1998) In vivo expression of therapeutic human genes for dopamine production in the caudates of MPTP-treated monkeys using an AAV vector. *Gene Therapy* **5**:820-827.

29. **Kaplitt MG**, Darakchiev BJ, During MJ (1998) Prospects for gene therapy in pediatric neurosurgery. *Pediatr Neurosurg* **28**:3-14.

30. During MJ, Xu R, Young D, **Kaplitt MG**, Sherwin RS, Leone P (1998) Peroral gene therapy of lactose intolerance using an adeno-associated virus vector. *Nat Med* **4**:1131-1135.

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Subthalamic GAD Gen Th rapy in a Parkinson's Disease Rat Model

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The motor abnormalities of Parkinson's disease (PD) are caused by alterations in basal ganglia network activity, including disinhibition of the subthalamic nucleus (STN), and excessive activity of the major output nuclei. Using adeno-associated viral vector–mediated somatic cell gene transfer, we expressed glutamic acid decarboxylase (GAD), the enzyme that catalyzes synthesis of the neurotransmitter GABA, in excitatory glutamatergic neurons of the STN in rats. The transduced neurons, when driven by electrical stimulation, produced mixed inhibitory responses associated with GABA release. This phenotypic shift resulted in strong neuroprotection of nigral dopamine neurons and rescue of the parkinsonian behavioral phenotype. This strategy suggests that there is plasticity between excitatory and inhibitory neurotransmission in the mammalian brain that could be exploited for therapeutic benefit.

Degeneration of specific groups of cells characterizes many neurological disorders. In PD, neurons of the substantia nigra pars compacta (SNc) are particularly vulnerable, leading to marked depletion of dopamine in the primary projection region, the striatum. As a result, the major inhibitory-output nuclei of the basal ganglia, the substantia nigra pars reticulata (SNr) and internal segment of the globus pallidus (GPi), are driven by a disinhibited and thereby overactive subthalamic nucleus (*1–4*) whose projection axons end in asymmetric, excitatory synapses on target neurons in the SNr (*5*). Marked improvement of the motor symptoms of PD occurs following either STN ablation (*6*, *7*), electrical inhibition with high-frequency stimulation (*8*, *9*), or pharmacological silencing by local lidocaine or muscimol infusion (*10*). Here, we describe a genetic approach to test the hypothesis that the glutamatergic neurons of the STN can be induced to express GAD, and thereby change from an excitatory nucleus to a predominantly inhibitory system that releases GABA at its terminal region in the substantia nigra (SN), leading to suppression of firing activity of these SN neurons. Moreover, we show that such an intervention also results in neuroprotection with resistance to 6-hydroxydopamine (6-OHDA)–induced degeneration of dopaminergic neurons.

We used recombinant adeno-associated virus (rAAV) to transduce neurons in the STN (*11*). This vector not only provides for highly efficient and stable gene transfer (*12*, *13*), but also results in minimal inflammatory and immunological responses (*14*). GABA, the brain's major inhibitory transmitter, can be generated by two isoforms of GAD, GAD65 and GAD67 (supporting text online). We generated rAAV vectors (*11*) containing both GAD65 and GAD67 cDNAs using the cytomegalovirus enhancer/chicken β-actin (CBA) promoter (*15*) and a woodchuck hepatitis virus postregulatory element (WPRE) (*16*) to further enhance expression (fig. S1A).

Mouse neural progenitor C17.2 cells were infected with both the GAD65 and GAD67 vectors with functional expression of the transgene confirmed by immunocytochemistry with antibodies specific to each GAD isoform and GABA (*11*) (fig. S1, B to J). GABA release was quantified by high-performance liquid chromatography (*11*, *17*) (fig. S1K).

Adult male rats were stereotactically injected into the left STN with GAD65, GAD67, or control GFP (green fluorescent protein) vectors. Four to 5 months after surgery, expression of the transgenes was determined by immunofluorescence (*11*). Robust expression confined to the STN was obtained for all transgenes (Fig. 1, A to I) with a nuclear halo in the GAD65-transduced neurons consistent with membrane-bound enzyme (Fig. 1F), whereas both GAD67 (Fig. 1I) and GFP (Fig. 1C) filled the cell soma completely. The STN neurons send a major projection to the SNr, with efferents also to

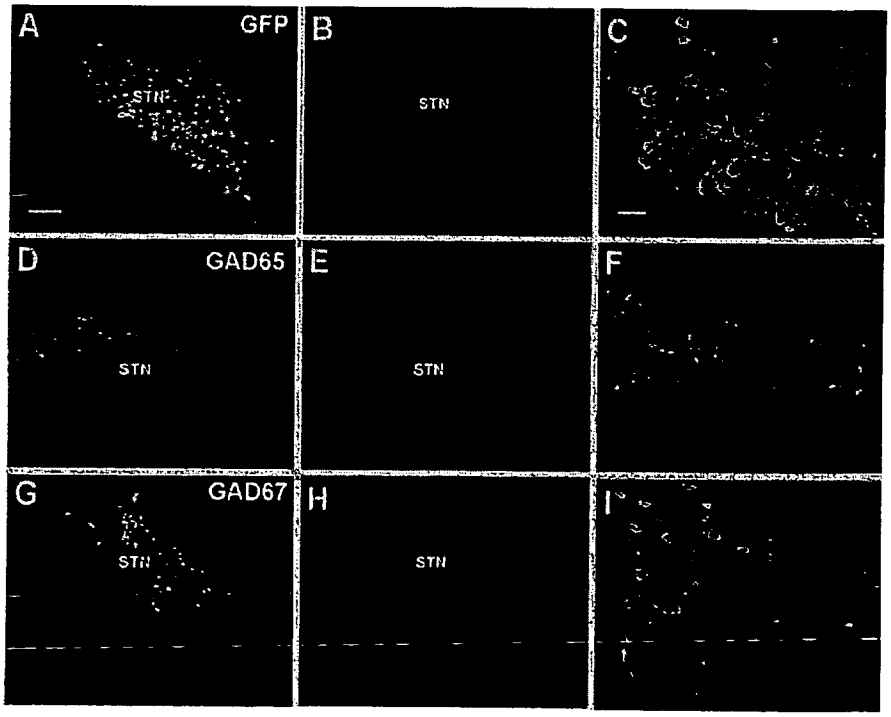

Fig. 1. rAAV-mediated transgene expression in the STN. AAV vectors were injected into the left STN. (A, D, G) Low-power ipsilateral (left) STN (bar, 100 μm). (B, E, H) Contralateral (C, F, I) High-power ipsilateral (bar, 30 μm). (A to C) GFP fluorescence. (D to F) GAD65 immunoreactivity. (G to I) GAD67 immunoreactivity.

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the SNc, as shown by the fiber staining in the rats expressing GFP, which acts as an anterograde tracer (Fig. 2, H to J). Sections were also analyzed with antibodies to CD4, CD8, major histocompatibility complex class I, and ED-1, a macrophage and microglial marker (fig. S2). In addition, sera were tested for the presence of antibodies to the AAV capsid or transgene (*18*). There was no significant immunoreactivity, humoral responses, or cellular infiltration in any animal, consistent with our previous report on lack of immunogenicity following rAAV injection into the mammalian brain (*14*).

To test the hypothesis that the expression of GAD in the STN induced by rAAV-mediated gene transfer induces a phenotypic shift, we assessed the responses of unlesioned control and 6-OHDA–lesioned parkinsonian rats that had received GAD65, GAD67, GFP, or saline 4 to 5 months earlier, using a combination of microdialysis and electrophysiology (*11*). Microdialysis probes were introduced into the SNr (Fig. 2, H and J), and a stimulating electrode implanted into the ipsilateral STN (Fig. 2, H and I). Samples were analyzed for glutamate and GABA (*11*). Representative data from individual unlesioned control rats (Fig. 2A), or from parkinsonian rats treated with either saline (Fig. 2B), GFP (Fig. 2C), GAD65 (Fig. 2D), or GAD67 (Fig. 2E), are shown, as well as the pooled data with means ± SEM of GABA (Fig. 2F) and glutamate concentrations (Fig. 2G). In the unlesioned control rats, as well as in saline- and GFP-treated parkinsonian rats, there was no significant increase in either GABA or glutamate release with STN stimulation. In contrast, following GAD65 gene transfer there was a (4.0 ± 1.5)-fold increase in GABA release associated with the STN stimulation ($P < 0.05$) (Fig. 2F).

A subgroup of rats had recording electrodes placed in the SNr, in addition to the STN stimulating electrode (*11*) (supporting text online). Single-unit recording from SNr cells during STN stimulation led to excitatory responses in 74% of SNr cells recorded in naïve unlesioned rats (Fig. 3A). This is consistent with the lack of GAD expression in the STN and the presence of glutamate immunoreactivity and asymmetrical synapses in the axon terminals (*5*, *19*). In parkinsonian rats, the responses of the SNr cells to STN stimulation were similar to that of unlesioned rats, with 83% showing excitatory responses ($P = 0.065$, Chi-square analysis); however, there was a more robust bursting response to each stimulation of the STN compared with the single-spike responses in naïve rats (Fig. 3B). GFP rats exhibited SNr responses that were indistinguishable from those of the saline parkinsonian rats, with 83% and 6% showing excitatory and inhibitory responses, respectively. The ratio of excitatory to inhibitory responses was markedly altered following GAD65 gene transfer (Fig. 3A). The excitatory single-unit recordings were reduced to 17% of responses in the GAD65 rats ($P < 0.0001$). In contrast, inhibitory responses observed in only 5, 10, and 6% of cells recorded in unlesioned, saline, and GFP parkinsonian rats, respectively, were increased to 78% in the GAD65 rats ($P <$ 0.001). In addition to the marked increase of inhibitory responses, the GAD65 rats also showed prolonged inhibition following STN stimulation, lasting 100 to 200 ms. Recordings from the GAD67-treated rats revealed a predominant excitatory response (62%) (Fig. 3). Inhibitory responses in GAD67 rats were in-

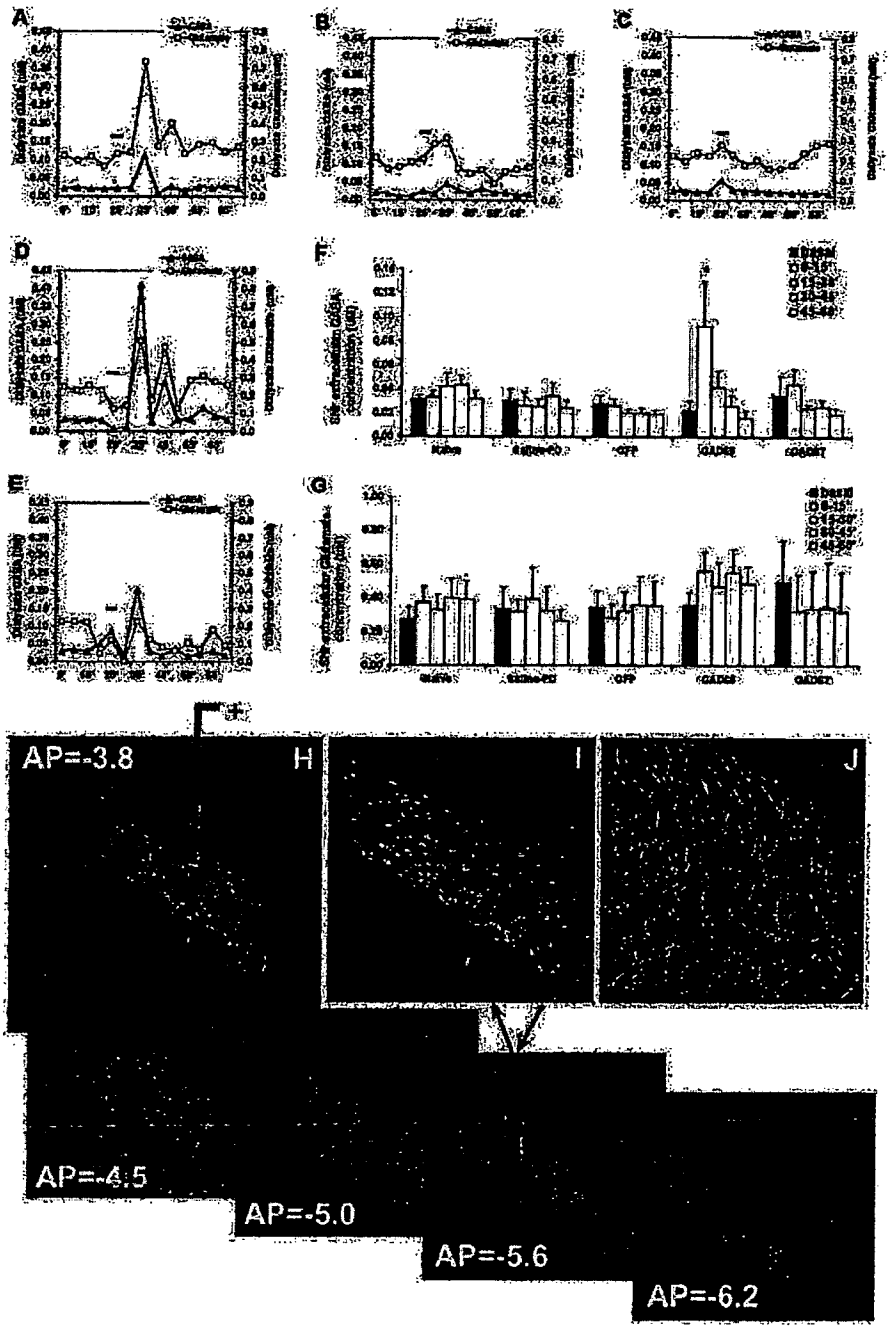

Fig. 2. Representation of electrode and dialysis probe implantation and SN microdialysis. (A to D) Sequential 5-min dialysate GABA and glutamate concentrations of a representative single animal in (A) unlesioned control rats, (B) saline, (C) GFP, (D) GAD65, and (E) GAD67 parkinsonian rats. The horizontal bar indicates the 5-min stimulation period. (F and G) Mean ± SEM of 15-min pooled data ($n = 4$ for each of the five groups) for GABA and glutamate, respectively. (H) Sequential sections through the midbrain of a GFP rat demonstrating the transduced STN (high power, I) and placement of stimulating electrode, and microdialysis probe lying in the SNr with high power (J) showing the intensity of fiber staining in the SN. Asterisk, $P < 0.05$, repeated measures ANOVA.

A

Group	Excitatory	Inhibitory	No Response
Control (n=5)	14 (74%)	1 (5%)	4 (21%)
Saline (n=5)	25 (83%)	3 (10%)	2 (7%)
GFP (n=3)	15 (83%)	1 (6%)	2 (11%)
GAD67 (n=4)	13 (62%)	7 (33%)	1 (5%)
GAD65 (n=4)	3 (17%)	14 (78%)	1 (6%)

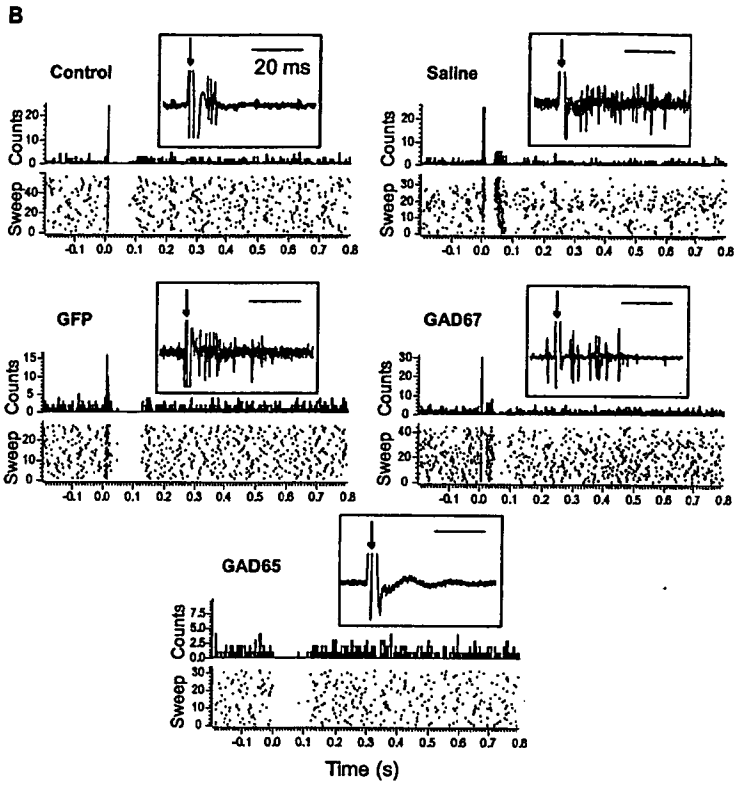

Fig. 3. (A) SNr electrophysiology in unlesioned control rats and saline-, GFP-, GAD65-, and GAD67-treated parkinsonian rats. Summary of the response of SNr neurons to electrical stimulation of the STN. The number of neurons that were excited, inhibited, or did not respond to STN stimulation are listed. The overall percentage of neurons in each category is in parentheses. (B) Histograms (2-ms bin width) and raster plots of SNr impulse activity in unlesioned control rats and saline-, GFP-, GAD65-, and GAD67-treated parkinsonian rats. In each case, the data shown are summarized from at least 30 presentations of the stimulus. All mean excitatory spike latencies were <7 ms from the onset of the stimulus, which is consistent with a monosynaptic connection of the STN to the SNr. (Insets) Electrophysiology traces from SNr neurons during STN stimulation are shown for each experimental group. Three overlay sweeps are shown. The arrowmark represents the stimulus artifact. The stimulus artifact is not included in the rasters and histograms because of the waveform matching program used selected only spikes for analysis (Spike 2, version 4; CED Inc., Cambridge, UK).

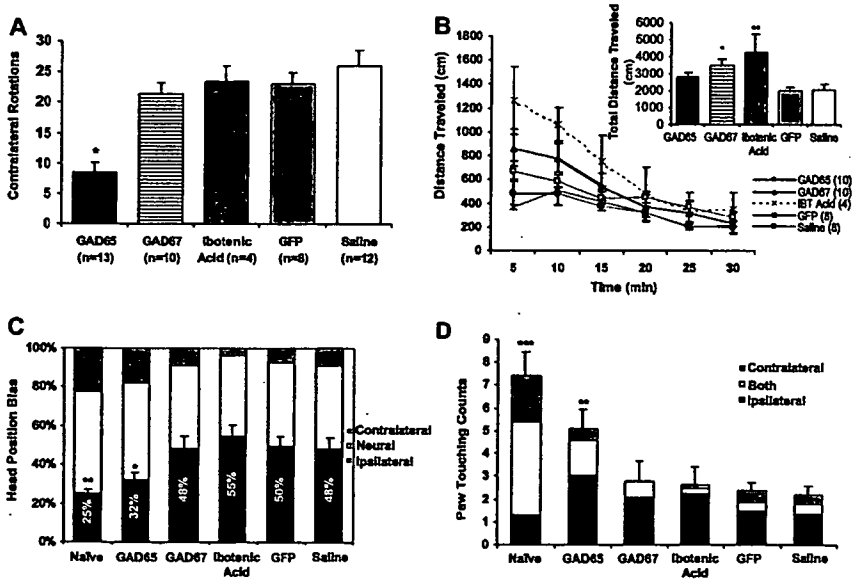

Fig. 4. GAD65 transduction of the STN inhibits 6-OHDA–induced parkinsonian asymmetry. (A) Apomorphine-induced contralateral rotations. (B) Locomotor activity. (C) Head position bias. (D) Paw touching behavior. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ANOVA with post hoc Fisher's PLSD test.

creased to 33% of all recordings ($P < 0.02$), consistent with an intermediate phenotype between the saline and GAD65 rats.

To further characterize the effects of the shift toward a mixed phenotype of the STN projection to the SNr, we examined whether the increased inhibitory tone might influence the ability of midbrain dopaminergic neurons to withstand a neurotoxic insult. Young adult male rats received intraSTN GAD65 (n = 13), GAD67 (n = 10), GFP (n = 8), saline (n = 12), or ibotenic acid (n = 4) (to further control for nonspecific lesion effects associated with the surgery and gene transfer). Three weeks after surgery, the ipsilateral (left) medial forebrain bundle (MFB) was lesioned with 6-OHDA (*11*). Outcome measures of the lesion severity and potential neuroprotection included analysis of behaviors dependent on an intact and symmetrical midbrain dopaminergic pathway. Both spontaneous and drug-induced behaviors were assessed at 8 to 16 weeks after 6-OHDA lesioning (*11*).

MFB lesions in naïve, GFP-, or saline-treated control animals leads to impaired general locomotor activity, specific deficits in contralateral limb use, and apomorphine-induced rotations contralateral to the denervated side. These rotations provide a highly reproducible and quantitative surrogate marker of the dopaminergic deficit (*20*). In the GAD65 rats, rotation rates were decreased by ~65% compared with both the saline and GFP control rats ($P < 0.05$) (Fig. 4A). Rotation rates in the ibotenic acid and GAD67 rats were unchanged from those of controls. Total locomotor activity was increased in ibotenic acid–lesioned ($P < 0.005$) and GAD67 ($P < 0.05$) rats, with a trend toward an increase in the GAD65 rats (P = 0.16) compared with controls (Fig. 4B). The head position bias test (*21*) showed marked asymmetry in the controls with an ipsilateral bias, which was almost completely normalized in the GAD65 rats ($P < 0.05$) but not altered significantly in the other groups (Fig. 4C). Similarly, forelimb use assessed by quantitative paw touch counting was improved only in the GAD65 rats ($P < 0.01$) (Fig. 4D). Examination of the midbrain in these rats with unbiased stereology for cell counting (*22*) revealed pro-

found nigral dopaminergic cell loss in the saline- and GFP-treated rats, with >99% loss of tyrosine hydroxylase (TH) immunoreactivity of the SNc for both groups and 93 ± 4% and 94 ± 4% loss in the ventral tegmental area (VTA) of saline- and GFP-treated rats, respectively (Fig. 5, A and B), compared with the contralateral side (Fig. 5C). Fluorogold (FG) was injected into the striatum 2 weeks before analysis, but after the 6-OHDA lesion (Fig. 5, middle panels) to provide definitive proof of neuronal degeneration in this model and not simply loss of dopaminergic phenotype as defined by TH immunoreactivity. Moreover, when administered after lesioning, intrastriatal FG injection with subsequent labeling of cells in the SN provides confirmation of intact surviving neurons retaining projections to the striatum. In the GAD65 rats, 35 ± 14% [$P < 0.01$, analysis of variance (ANOVA) with Fisher's post hoc test versus GFP-treated rats] and 80 ± 11% ($P < 0.0001$) of dopaminergic neurons survived in SNc and VTA, respectively (Fig. 5, D and E). For the GAD67 rats, there was no significant protection of SNc neurons, with less than 1% survival, but there was a 42 ± 3% ($P < 0.02$) survival of VTA neurons (Fig. 5F). The ibotenic acid–treated rats had no protection of the SNc, but showed a trend toward protection of the VTA, with 19 ± 9% surviving neurons compared with the 6 ± 4% and 7 ± 4% surviving VTA neurons in the GFP- and saline-treated groups, respectively. Although at least one study has suggested dopaminergic neuroprotective efficacy of STN lesions (*23*), these investigators used intraSTN kainic acid and a less severe, partial intrastriatal 6-OHDA lesion model. In contrast, others have shown either no neuroprotection conferred by a quinolinic acid STN lesion (*24*), or a weak 23% neuroprotective effect of intraSTN ibotenic acid in the partial intrastriatal 6-OHDA lesion model (*25*). Thus, the marginal efficacy of a STN lesion (confined to the A10 cells in the VTA and not reaching statistical significance) in a much more severe MFB 6-OHDA lesion model suggests that the robust nigral neuroprotection we observed with GAD65 is not just due to a drop in the excitatory drive from the STN, but that the inhibitory neurotransmission in this pathway induced by GAD65 gene transfer is critical.

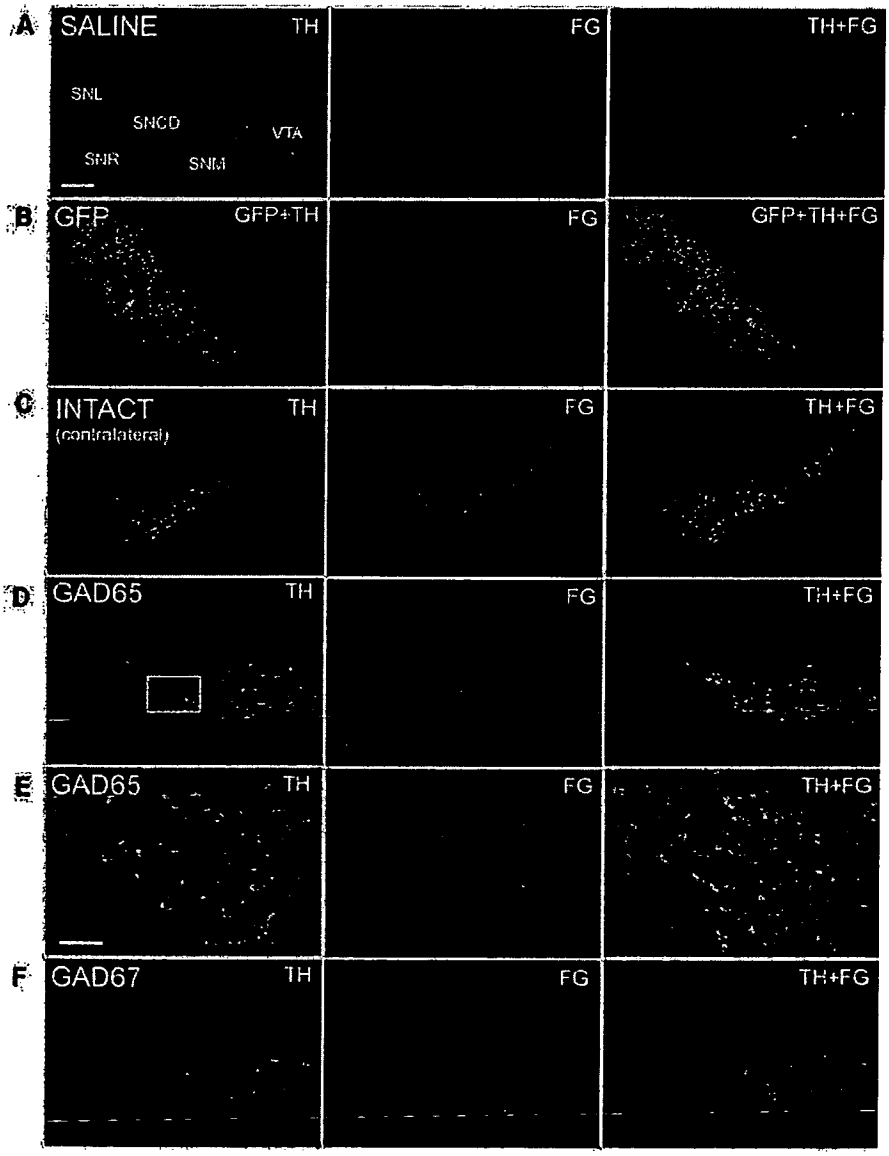

Fig. 5. GAD65 mediates increased survival of SN and VTA tyrosine hydroxylase (TH)–positive neurons. TH immunofluorescence and fluorogold (FG) double-labeled images show survival of TH neurons. TH alone (left), FG (middle), and combined TH and FG (right). (A) Lesioned hemisphere of a representative saline-injected parkinsonian rat. (B) GFP. (C) Representative intact contralateral hemisphere. (D) Low-power and (E) high-power GAD65. (F) GAD67. SN, substantia nigra; SNM, medial SN; SNCD, dorsal pars compacta SN; SNL, lateral SN; SNR, pars reticulata SN. Bar, 200 μm [(A) to (D) and (F)], 50 μm (E).

Although in our study we used gene transfer to elicit the phenotypic shift, it has been previously shown that certain groups of neurons generally considered excitatory and glutamatergic can also express GAD transcripts. Specifically, hippocampal dentate granule and CA1 cells express very low levels of GAD65 and GAD67 mRNA (*26*). Moreover, these cells can also express low levels of the protein, but increase expression with electrical stimulation (*27*) or following seizures (*28*, *29*). Hence, there is plasticity with the potential for heterotransmission of an inhibitory transmitter in well-characterized excitatory pathways in pathophysiological states.

The success of STN deep-brain stimulation and subthalamotomy for patients with advanced PD, together with symptomatic relief mimicked by infusion of the $GABA_A$ agonist muscimol into the STN (*10*) or directly into the SNr in Parkinsonian monkeys (*30*), suggests that a gene-transfer strategy that enhances GABA transmission in the STN and its terminal regions may be similarly effective. The limited efficacy of intraSTN ibotenic acid to protect against a subsequent MFB 6-OHDA lesion suggests that GAD65 gene transfer may be more effective than simple ablation or local electrical silencing. At present, there are no treatments for PD shown to definitively attenuate disease progression. Although our data suggest some promise, both rodent and nonhuman primate studies are insufficient to predict neuroprotection in the clinic. Such an answer will require large blinded clinical trials and will be the ultimate goal of such a gene-transfer approach.

Our data, including behavior, immunohistochemistry, in vivo neurochemistry, and single unit–recording electrophysiology, are strongly supportive of the concept of hetero-

transmission and inherent plasticity of the mammalian nervous system, with transfer of a single gene in a specific population of cells leading to a marked phenotypic change from largely excitatory to predominantly inhibitory transmission. Whether this shift induces additional phenotypic changes including ultrastructure typical of inhibitory neurons will be a focus of future studies. GAD gene transfer into glutamatergic excitatory neurons leading to an inhibitory bias with altered network activity and a neuroprotective phenotype holds potential for treatment of PD and other neurological conditions associated with excessive excitation.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/298/5592/425/DC1
Materials and Methods
SOM Text
Figs. S1 and S2
References and Notes

30 May 2002; accepted 2 August 2002

SEPTEMBER 24, 2001

Developments to Watch

Edited by Otis Port

Printer-Friendly Version
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Gila Monsters: Can They Sharpen Your Memory?

Memories are made of many things, and one of them may soon be Gila monster spit. Dr. Matthew During, a neuroscientist at Philadelphia's Thomas Jefferson University, has discovered that a peptide found in the saliva of these lizards can dramatically improve the memories of rats. The synthetic version, dubbed Gilatide, has been licensed to Axonyx Inc. in New York, which hopes the drug will be the first to successfully boost learning and memory, as well as provide help for people suffering from attention deficit disorder and age-related memory loss.

During's inspiration stems from research showing that various peptides released in the gut interact with memory receptors in the brain. This is probably an evolutionary adaptation that helped animals remember where they caught a tasty evening meal. He studied the saliva of Gila monsters because lizards sense prey with their tongues. Colin N. Haile, an assistant professor who works with During, says Gilatide produces impressive benefits in helping rats to remember how to run a maze. "Just one administration of the drug resulted in very potent memory retention," which may last as long as 21 days, he says. Rats aren't people. But Gilatide could enter human clinical trials in two years.
By Catherine Arnst

BACK TO TOP

On the Trail of "Reversible" Polymers

Towering piles of plastic containers and toys at landfills testify to the durability of polymers. Once the links are formed in the long chains of molecules that make up these materials, it's almost impossible to break the bonds and retrieve the original compounds. But by custom-designing

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Weill-CU/Auckland study shows efficacy of gene therapy for Parkinson's

By Jonathan Weil

In a study published Oct. 11 in the journal *Science*, scientists from the University of Auckland, New Zealand, and Weill Cornell Medical College report on the effectiveness of a new gene therapy approach to Parkinson's disease and the potential for this therapy to affect the overall progression of the disease itself. Based on this study and other data, the U.S. Food and Drug Administration (FDA) has given its approval to begin testing the therapy in a small Phase I clinical trial. This will be the first time that gene therapy will be used in patients with Parkinson's disease.

The *Science* article is authored by lead investigator Dr. Matthew J. During, professor of molecular medicine at the University of Auckland, first author Dr. Jia Luo and co-investigator Dr. Michael G. Kaplitt, director of stereotactic and functional neurosurgery and assistant professor of neurological surgery at Weill Cornell Medical College. During and Kaplitt are also co-principal investigators on the upcoming clinical trial of this therapy.

Researchers Dr. Matthew During, left, professor of molecular medicine at the University of Auckland, and Dr. Michael Kaplitt, director of stereotactic and functional neurosurgery and assistant professor of neurological surgery at Weill Cornell Medical College. *Photo by Alan R. Arellano*

"We are using gene therapy to 'reset' a specific group of cells that have become overactive in an affected part of the brain, causing the impaired movement and other symptoms associated with Parkinson's disease," said During. "We are very encouraged that in addition to the effect this therapy has on quieting symptoms, we present evidence that suggests it may arrest or delay disease progression."

People with Parkinson's disease have a profound loss of a specific group of nerve cells deep in the brain that make dopamine, a signaling molecule. The loss of dopamine leads to a disturbance in the brain's network activity controlling movement. In the center of this network is a region called the subthalamic nucleus (STN), which in Parkinson's disease is extremely overactive and, if silenced, leads to a dramatic reduction in the symptoms.

Targeting the overactive cells, researchers inserted the GAD gene into a viral vector (adeno-associated virus or AAV) to allow it to be efficiently delivered into the affected region of the brain. GAD is responsible for making a small molecule called GABA, which is released by nerve cells to inhibit, or dampen, activity. After this gene therapy is introduced, the overactive cells are "reset," and brain network activity controlling movement returns towards more normal function. In 1994, Kaplitt and During were the first to demonstrate that AAV could be a safe and effective vehicle for gene therapy in the brain. Since that time, AAV has been used safely in a variety of clinical gene therapy trials, and the virus has never been associated with any human disease.

Although medical therapy is usually effective for most symptoms of Parkinson's, over time many

patients become resistant to treatment or develop disabling side effects.

"Current surgical therapies for such patients attempt to interrupt this network abnormality by destroying overactive brain areas or placing DBS (deep brain stimulation) electrodes to quiet these areas. Both of these treatments, however, have certain limitations and side effects," said Kaplitt. "Our approach is based on a similar rationale, but we use gene therapy to adjust the chemical signaling of these brain areas to a more normal setting. This exploits the best parts of current therapy but makes it more powerful, less invasive and potentially safer."

The *Science* paper reports on testing of this theory using a combination of techniques to measure brain function in rodents that were made Parkinsonian. Five tests were conducted. These tests showed the GAD gene was present and producing GABA as anticipated. Several behavior tests also were conducted in the rodents to show they had retained more normal function and did not develop further signs of Parkinson's as did the control rats. Primate studies also demonstrated that the therapy was safe and there were no toxicities associated with the treatment.

Based upon the recent FDA approval, the first Phase I trial of this treatment is anticipated to begin within the next few months. Selected patients will undergo surgical gene therapy by Kaplitt at NewYork-Presbyterian Hospital/Weill Medical College of Cornell. These patients will be recruited and followed by Drs. David Eidelberg and Andrew Feigin at the North Shore Hospital Long Island Jewish Movement Disorder Clinic. The initial trial will be limited to 12 patients with severe Parkinson's disease of at least five years duration, for whom current therapies are no longer effective.

"The *Science* report and our team's translation of this approach to treatment of human disease represent the culmination of over a decade of research in this area," said During. "Our primary objective has been to stress patient safety above all else, and the NIH [National Institutes of Health] and FDA have helped us design a clinical intervention which has exciting efficacy potential and attempts to minimize in any way possible risks of adverse events to patients in the study. It is our hope that, with this approach, our trial will help demonstrate that gene therapy in the brain can be both safe and effective."

Other contributing authors include Helen Fitzsimons, David S. Zuzga and Yuhong Liu from the Functional Genomics and Translational Neuroscience Laboratory, Department of Molecular Medicine and Pathology, University of Auckland, and Michael L. Oshinsky from the CNS Gene Therapy Center, Jefferson Medical College, Philadelphia.

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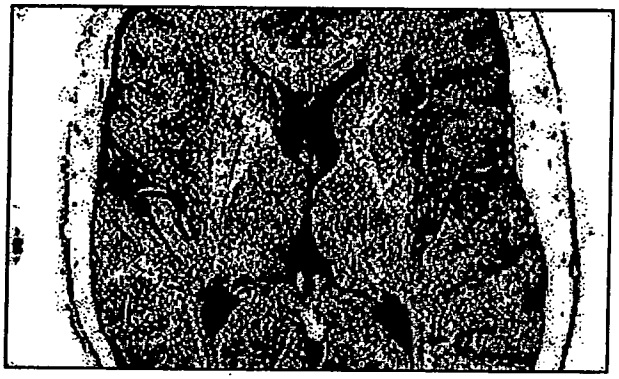
Thursday, 10 October, 2002, 17:55 GMT 18:55 UK

Gene therapy for Parkinson's



The treatment is delivered directly to the brain

Doctors are to begin the first ever trial using gene therapy to treat Parkinson's Disease.

They hope gene therapy will be more effective and less invasive than the medical or surgical treatments currently available, which can be linked to side effects.

And they say it may even be possible to halt the progress of the incurable degenerative condition.

Around 120,000 people in the UK have Parkinson's.

The techique uses gene therapy to "re-set" a specific group of cells in the brain which have become overactive.

> **"It may arrest or delay disease progression"**
>
> **Dr Matthew During, University of Auckland**

This causes the impaired movement and other symptoms associated with Parkinson's.

The team of scientists, from New Zealand and the US have carried out successful tests in rats, and hope to begin the first trials in humans by the end of the year.

Twelve patients who have had severe

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Parkinson's for at least five years, and for whom current therapies are no longer effective, will take part.

Movement control

Patients with Parkinson's Disease have too few of a specific group of nerve cells deep in the brain which make the signalling molecule dopamine, which affects how the brain controls movement.

A region at the centre of this network, called the subthalamic nucleus (STN), becomes extremely overactive in patients with Parkinson's.

If this can be silenced, patients see a dramatic reduction in their symptoms.

> **"It is very encouraging for people with Parkinson's"**
>
> **Robert Meadowcroft, Parkinson's Disease Society**

The gene therapy uses the GAD gene, which makes a small molecule called GABA. This is released by nerve cells to inhibit, or dampen activity.

Scientists deliver this directly to the overactive cells by inserting the GAD gene into a modified virus.

This gene therapy "re-sets" the overactive cells, and brain activity becomes more normal. normal.

Tests on rats showed the GAD gene was present and producing GABA as anticipated.

Behaviour tests showed they had retained more normal function and did not develop further signs of Parkinson's compared to rats who had not been given the gene therapy.

In addition to work in rats, tests on monkeys have shown the therapy was safe and non-toxic.

Chemical signals

Dr Matthew During, professor of molecular medicine at the University of Auckland, who is leading the research, said: "We are very

encouraged that in addition to the effect this therapy has on quieting symptoms, we present evidence that suggests it may arrest or delay disease progression.

Follow researcher Dr Michael Kaplitt, a specialist in neurosurgery at Weill Cornell Medical College, New York, said: "Both surgical and medical treatments have certain side effects.

"We use gene therapy to adjust the chemical signalling of these brain areas to a more normal setting.

"This exploits the best parts of current therapy but makes it more powerful, less invasive and potentially safer."

Robert Meadowcroft, director of policy, research and information at the UK's Parkinson's Disease Society, told BBC News Online: "I think it is very encouraging for people with Parkinson's that this trial is going forward.

"In terms of conventional treatment for Parkinson's, most people will have drug treatment.

"In the first stages, that can be very effective. But after four or five years the side effects, such as dyskinesias, or unwanted movements, can become quite distressing themselves."

He said surgery would only be suitable for a small proportion of Parkinson's patients.

Mr Meadowcroft added: "This research sets out some very promising findings from animal studies, and therefore we look forward with hope, but caution."

The research is published in the magazine Science.

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In This Section Go

Stereotactic Gene Based Hypothalamic Neuromodulation

Meeting: AANS 2002 Chicago

Presentation type: Oral

Authors: Nicholas M Boulis, MD, ; Aaron J Noordmans, BS; Debra K Song, BS; Paola Leone, PhD; During Matthew, PhD

Abstract:
Introduction: Gene based approaches to stereotactic neuromodulation provide potential for elegant synaptic regulation through manipulation of proteins that underlie transmission. The ability to induce long lasting gene expression within stereotactic brain targets without structural damage has been achieved using adeno-associated viral vectors (rAAV). In the current project, this strategy was used to alter rat metabolic behaviour through gene based augmentation of GABA production in the lateral nucleus of the hypothalamus (LH).
Methods: Two rAAV-2 vectors containing the genes for green fluorescence protein (GFP) or glutamate decarboxylase (GAD), the CAG promoter and the woodchuck polyribosomal enzyme (WPRE) sequence were constructed (CNS Gene Therapy Center, Thomas Jefferson University). 500 nl of viral solution (10e12 pt/ml) or 0.9% saline control was stereotactically injected into the LH bilaterally. Animals received daily weight, rat chow consumption, urine, and feces measurements for 1 week prior to surgery and 2 weeks after injection. WPRE in situ hybridization and GFP fluorescence analysis was conducted at 3 weeks.
Results: LH contained 0.25 cubic mm of GFP expression bilaterally. Mean targeting errors were 0.37+/-0.18 mm AP, 0.18+/-0.12 mm SI, 0.49+/-0.19 mm Med-Lat. In situ hybridization revealed persistence of the rAAVGAD sequence in the LH 3 weeks following injection. Animals in control (rAAVGFP and saline) groups showed 30 gram weight gain between postoperative weeks 1 and 2 in contrast to the rAAVGAD group which had a 20 gram weight reduction ($p<0.05$). These changes in weight were paralleled by a reduction in food intake in the rAAVGAD group compared to increased food intake in control groups ($p<0.03$). No significant differences were detected in feces or urine production. Conclusions: Accurate stereotactic delivery of rAAV vectors can alter gene expression in deep brain targets in a sustained fashion. Transgene specific local inhibition is possible through gene based enhancement of GABA production

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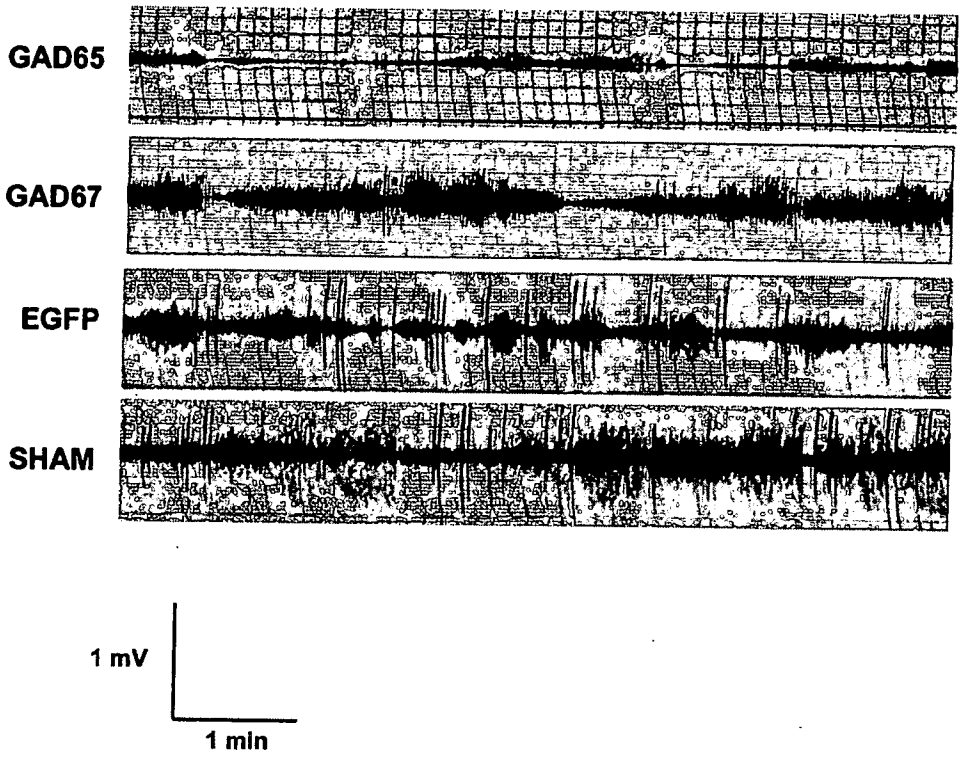

Figure :1

Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex

Luc Jasmin*†, Samuel D. Rabkin‡, Alberto Granato§, Abdennacer Boudah* & Peter T. Ohara†

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It is known that pain perception can be altered by mood, attention and cognition, or by direct stimulation of the cerebral cortex[1], but we know little of the neural mechanisms underlying the cortical modulation of pain. One of the few cortical areas consistently activated by painful stimuli is the rostral agranular insular cortex (RAIC) where, as in other parts of the cortex, the neurotransmitter γ-aminobutyric acid (GABA) robustly inhibits neuronal activity. Here we show that changes in GABA neurotransmission in the RAIC can raise or lower the pain threshold—producing analgesia or hyperalgesia, respectively—in freely moving rats. Locally increasing GABA, by using an enzyme inhibitor or gene transfer mediated by a viral vector, produces lasting analgesia by enhancing the descending inhibition of spinal nociceptive neurons. Selectively activating $GABA_B$-receptor-bearing RAIC neurons produces hyperalgesia through projections to the amygdala, an area involved in pain and fear. Whereas most studies focus on the role of the cerebral cortex as the end point of nociceptive processing, we suggest that cerebral cortex activity can change the set-point of pain threshold in a top-down manner.

It is well known that painful stimuli activate the insular cortex[2], but less is known of how the output of the insular cortex might alter pain. Studies in humans and animals show that inhibition or lesion of this cortical area produces analgesia[3–5], indicating that the insular cortex tonically produces hyperalgesia. Tonic depression of the nociceptive threshold could result from the activation of pro-nociceptive areas of the brain or from inhibition of the endogenous pain inhibitory system[6]. To examine the relationship between cortical activity and nociceptive threshold, we manipulated GABA neurotransmission in the RAIC and then tested nociceptive responses with the heat paw-withdrawal test. GABA levels were increased either by inhibiting GABA-aminotransferase-mediated degradation[7] using vigabatrin, or novel expression of glutamic acid decarboxylase 67 (GAD-67), one of the enzymes responsible for synthesizing GABA from glutamate.

Increasing GABA concentration unilaterally in the RAIC with vigabatrin (78 nmol in 200 nl), resulted in a clear and consistent bilateral analgesia, an effect not seen when the injections were in the surrounding brain areas (Fig. 1a, b). Injection of the $GABA_A$ antagonist bicuculline (200 pmol/200 nl) in the RAIC after vigabatrin normalized the nociceptive threshold, indicating that the effect of vigabatrin was receptor-mediated. Injection of the sodium-channel blocker bupivicaine (200 nl of a 0.25% solution) in the RAIC yielded similar results to vigabatrin (Supplementary Fig. 1a). The antinociceptive effect of vigabatrin seemed specific, because no motor or behavioural impairment was revealed in the open-field (no difference between vigabatrin-treated and saline-treated for any of the parameters; $n = 6$ per group, $P > 0.05$) and rotarod tests (Supplementary Fig. 1b). To determine whether a sustained increase in GABA would produce long-term analgesia, we injected the defective herpes simplex virus (HSV) vector dvGAD-67 (5×10^3 defective particle units/1.5 μl) encoding GAD-67. Infection of both neuronal and glial cells with dvGAD-67 leads to a continued synthesis and release of GABA[8,9] and resulted in bilateral antinociception (Fig. 1c, d) lasting for up to 10 days. Motor and general behaviours were unaltered (Supplementary Fig. 1b–d). Histological analysis confirmed that local expression of the LacZ reporter gene encoding β-Galactosidase (Fig. 1d) was confined to the RAIC and, with the exception of an occasional cell being labelled along the cannula tract, no labelling was found in other brain areas. Injection of a control viral vector expressing only LacZ and alkaline phosphatase had no effect (Fig. 1c), indicating that antinociception did not result from a non-specific event related to the injection of viral vectors.

The above behavioural results demonstrate that GABA-mediated neurotransmission is directly involved in the pain modulatory function of the RAIC. The long duration of the effect obtained with dvGAD-67 suggests that the antinociceptive action occurs through neural mechanisms that do not downregulate over time.

To determine whether the local increase in cortical GABA concentration changed the nociceptive threshold by an action on the descending pain inhibitory system[6], we administered the non-selective α-adrenoreceptor antagonist phentolamine (3 nmol in 10 μl) intrathecally over the lumbo-sacral spinal cord through a chronically implanted catheter[10]. Phentolamine blocks descending inhibition mediated by noradrenergic bulbo-spinal projections, most of which originate from the locus coeruleus[11,12]. In all cases phentolamine reversed the antinociception induced by the increased cortical GABA concentration (Fig. 1e), indicating that the antinociceptive effect of inhibiting the RAIC involves an increased activity of noradrenergic bulbo-spinal projections. The dose of phentolamine used here was too low to alter the baseline nociceptive threshold (Fig. 1e).

We then examined projections from the RAIC that might modulate noradrenergic bulbo-spinal neurons. The RAIC was found to have bilateral projections to GAD-immunopositive cells in the caudal brainstem (Fig. 2a–d), including GABAergic cells in nucleus raphe magnus (Fig. 2c, d), many of which project to the locus coeruleus[13]. On the assumption that the output of the RAIC is glutamatergic, the inhibitory effect of RAIC activity on the locus coeruleus is probably mediated by the activation of GABAergic neurons contacted by RAIC afferents (Fig. 2a–d). Indeed, up to two-thirds of afferents onto locus coeruleus neurons are GABAergic[13] and, together with opioidergic and catecholaminergic afferents, GABAergic afferents constitute the main inhibitory input[13]. It is therefore likely that the analgesia seen after increasing GABA in the RAIC results from a decreased activity of cortical afferents to the peri-coerulear inhibitory neurons, leading to a net increase in the activity of noradrenergic bulbo-spinal neurons (see Fig. 4 for a summary diagram).

GABA acts on neurons through two different types of receptor. In the RAIC, $GABA_B$ receptors are concentrated on pyramidal neurons of cortical layer 5 (Fig. 2e, f), whereas $GABA_A$ receptors are located diffusely throughout all cortical layers on dendrites and soma (Fig. 2g). Because most lamina V neurons are projection neurons, we used retrograde and anterograde tracing to locate their targets. We found that a large contingent of RAIC neurons expressing $GABA_B$ receptors project to the ipsilateral basolateral nucleus of the amygdala (45.1 ± 3.9%), and few $GABA_B$ neurons project to the peri-coerulear area.

These anatomical findings led us to propose that increasing the GABA concentration in the RAIC and then blocking $GABA_B$ receptors would selectively disinhibit projections to the amygdala, leaving other outputs silent and enabling us to determine the net effect of the disinhibited neurons on pain behaviour. The $GABA_B$-selective antagonist saclofen (100 nmol/200 nl; see Supplementary

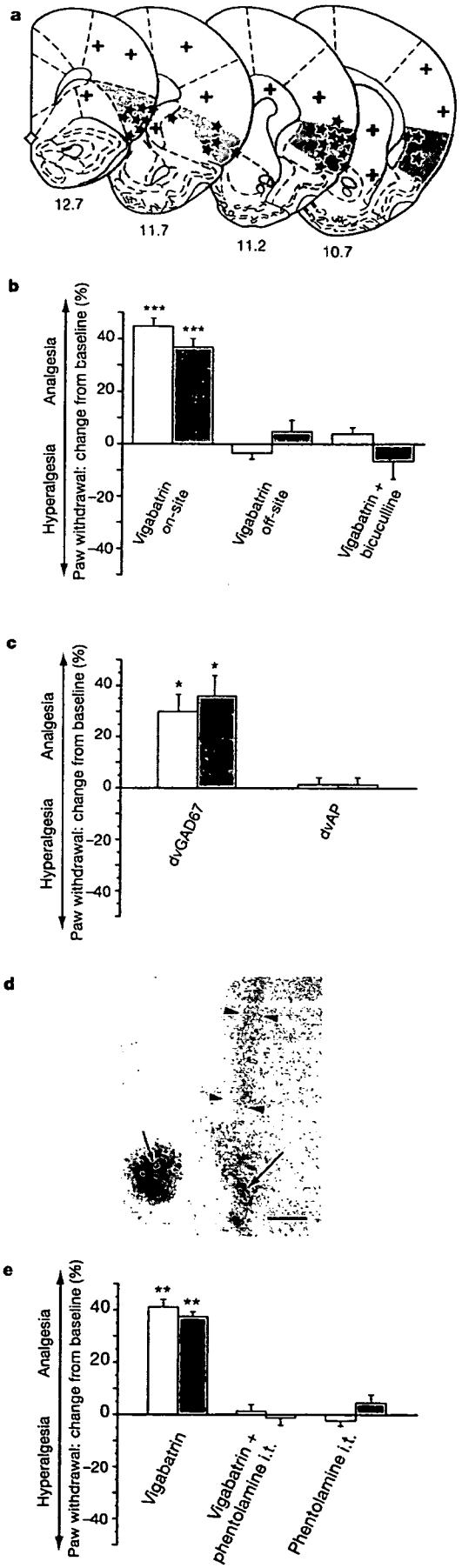

Fig. 2a for a dose–response curve) was microinjected into the RAIC 2 h after vigabatrin or 2–3 days after injection of dvGAD-67; the nociceptive threshold was then assessed. Rats that had become analgesic from vigabatrin or GAD-67 gene expression developed hyperalgesia, principally ipsilateral, immediately after the injection of saclofen (Fig. 3a, b). Fos immunocytochemistry confirmed increased activity in the amygdala (Figs 2h, i and 3c) associated with disinhibition of the $GABA_B$ cortical projection cells and the resulting hyperalgesia. Injection of saclofen alone into the RAIC did not alter the nociceptive threshold (Fig. 3b), indicating that the hyperalgesic effect of disinhibiting $GABA_B$-receptor-bearing neurons in the RAIC might normally be opposed by other, unknown, neurons bearing $GABA_A$ receptors only.

We then determined that projections from the RAIC to the amygdala, rather than to the caudal brainstem, have a key function in the hyperalgesic state. Bupivicaine (0.25%, 200 nl) injected into the basolateral nucleus of the amygdala after injections of vigabatrin and then saclofen into the RAIC, completely abolished the hyperalgesia, and the nociceptive threshold reverted to the same analgesic state obtained after the injection of vigabatrin alone (Fig. 3a). Ensuing intrathecal injection of phentolamine (3 nmol in 10 μl) returned the nociceptive threshold from analgesic to normal (Fig. 3a). In contrast, injection of bupivicaine in the locus coeruleus did not alter RAIC induced hyperalgesia (Supplementary Fig. 2b), and blocking the amygdala with bupivicaine had no effect on the analgesia induced by vigabatrin in the RAIC (Fig. 3b).

The RAIC therefore acts on at least two independent subcortical systems to modulate the nociceptive threshold (Fig. 4). The first, from the RAIC to the locus coeruleus, influences the noradrenergic bulbo-spinal projections and is modulated predominantly by $GABA_A$ receptors. The second, from the RAIC to the amygdala, is modulated predominantly by $GABA_B$ receptors. Because $GABA_B$ receptors are metabotropic, their function is probably to keep cortical afferents to the amygdala under prolonged inhibition. However, the effect of $GABA_B$ receptor stimulation is conditional on a number of factors such as the resting potential and concentrations of intracellular chloride[14], factors that significantly influence the outcome of GABA stimulation for cells expressing both types of receptor.

As the nociceptive threshold is reset by blocking RAIC activity with bupivicaine or by locally increasing the GABA concentration, this indicates that both of the above systems might be tonically active. However, tonic GABA release seems to contribute only moderately to the baseline activity of these projections because local injection of bicuculline alone did not significantly alter the nociceptive threshold (Supplementary Fig. 2c). This would imply that a lack of cortical GABA is unlikely to account for states of

Figure 1 Cortical injection sites and nociceptive heat paw-withdrawal responses. **a**, Drawings showing the location of the RAIC (shaded area) and representative on-site (asterisks) and off-site (plus signs) injections. Numbers represent rostro-caudal coordinates. **b**, On-site injection of vigabatrin produced a bilateral increase in withdrawal latency, whereas off-site injections had no effect. There was no statistical difference between left (white bars) and right (grey bars) paws. Injection of bicuculline restored the nociceptive threshold to normal. **c**, Injection of GAD-67-expressing vector (dvGAD-67; $n = 6$) produced bilateral analgesia, whereas injection of the control vector expressing alkaline phosphatase (dvAP) produced no behavioural effect. White bars, left paw; grey bars, right paw. **d**, β-Galactosidase-stained section of dvGAD-67-injected RAIC, showing infected cells (arrows) adjacent to the cannula tract (arrowheads). **e**, Analgesia induced by vigabatrin in the RAIC was reversed by intrathecal (i.t.) phentolamine. Phentolamine alone had no effect. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$. Scale bar in **d**, 100 μm.

hyperalgesia. In fact, a dysfunction of the noradrenergic system, which is a target of the RAIC, probably has a minor function in pain related to chronic inflammation or neuropathic injury, because the complete removal of this system does not, in the long term, alter the nociceptive threshold[15]. The direct projections from the RAIC to the caudal brainstem serotoninergic cell groups could mediate some of the pain-modulatory effects of the RAIC, including the hyperalgesia[16].

Our data indicate that if the RAIC is implicated in chronic pain it is through projections to the amygdala, the latter being part of a pro-nociceptive (pain-facilitating) circuit[17]. The preferential ipsilateral hyperalgesia after the injection of vigabatrin followed by

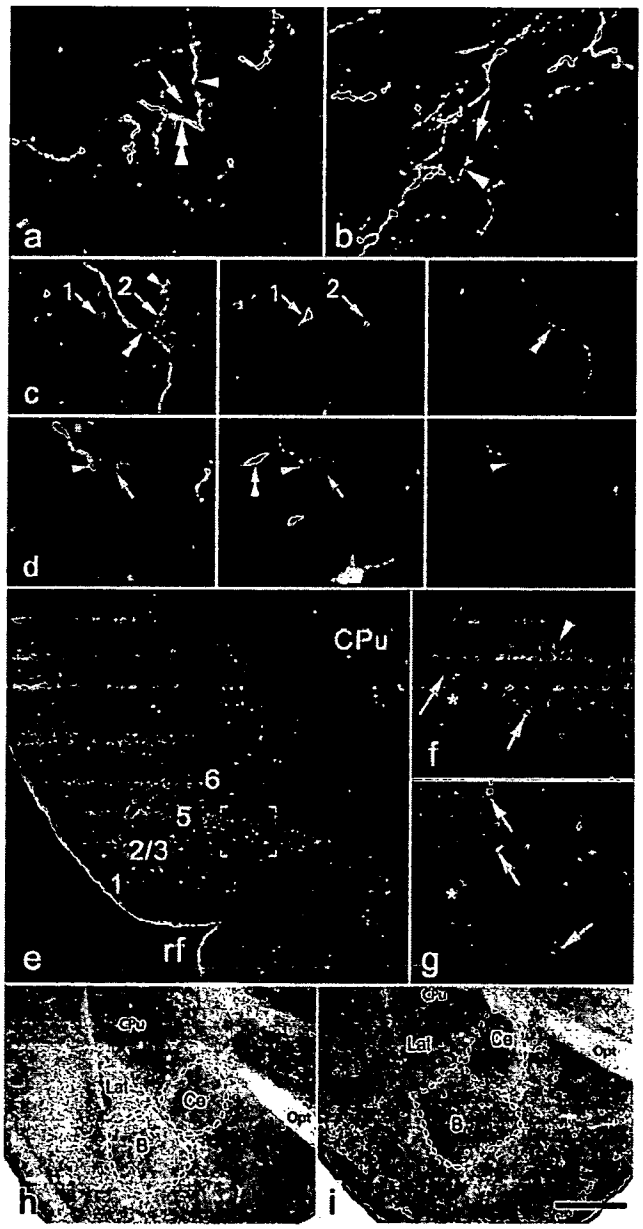

Figure 2 Immunocytochemistry of the RAIC, amygdala and brainstem. **a**, **b**, Confocal images of GAD-67-immunopositive cells (red, arrows) near the locus coeruleus (a) and parabrachial nucleus (b) in apposition to labelled axons (green, arrowheads) after injection of biotin–dextran (BDA) into the RAIC. **c**, Nucleus raphe magnus GABAergic neurons (left, arrows 1 and 2) double-labelled with Fluoro-Gold (middle, arrows 1 and 2) from locus coeruleus injection, in apposition to a BDA-labelled fibre from the RAIC (left and right, double arrowhead). Single arrowhead, GABA-only-labeled neuron. **d**, High magnification of a nucleus raphe magnus GABA cell (left, arrow) double-labelled with Fluoro-Gold (middle, arrow) from the locus coeruleus in apposition to puncta from a BDA-labelled fibre (right, arrowhead) from the RAIC. The BDA fibre is also labelled green (left) and is seen faintly as bleed-through fluorescence in the middle panel (arrowhead). Asterisk in left panel, single-labelled GABA-immunoreactive neuron; double arrowhead in middle panel, single-labelled Fluoro-Gold neuron. **e**, $GABA_B$-immunopositive pyramidal neurons in lamina 5 of the RAIC. 1–6, cortical layers; rf, rhinal fissure. **f**, $GABA_B$-immunostained neurons from area in **e** indicated by corners. Outer border (arrows) and inner border (arrowhead) of lamina 5 are indicated. **g**, $GABA_A$-receptor immunostaining of the same area as **f**. Arrows indicate labelled cell bodies, asterisk indicates the same blood vessel as in **f**. **h**, Fos immunocytochemistry of the amygdala after injection of vigabatrin into the RAIC. **I**, After disinhibition of $GABA_B$ neurons in the RAIC there was an increased number of Fos-immunopositive cells in the basolateral nucleus of the amygdala (B) and to a smaller degree in the central nucleus (Ce). CPu, caudate putamen; Lat, lateral nucleus of the amygdala; Opt, optic tract. Scale bar, 40 μm for **a**, **b**; 110 μm for **c**; 40 μm for **d**; 350 μm for **e**; 175 μm for **f**, **g**; 1.4 mm for **h**, **i**.

saclofen into the RAIC is not surprising, given previous reports that the amygdala affects morphine analgesia for the ipsilateral hemibody[18]. The present results indicate that the amygdala can enhance nociceptive responses in addition to its role in opiate analgesia[18–20] and learned fear[21]. Anaesthesia of the amygdala alone did not alter the nociceptive threshold (Supplementary Fig. 2d), confirming previous reports[18,21] and showing that the amygdala does not function independently as a 'pain generator' but seems to be controlled directly by cortical afferents. The amygdala is a nodal point where reflexive and learned responses to threatening stimuli are orchestrated. The basolateral nucleus, to which the RAIC has a major projection, is involved in instrumental responses or operant conditioning[22,23]. In the present protocol, animals were conditioned to interrupt an applied heat stimulus by lifting their paw, a characteristic instrumental response. Rats did not display any signs of stress, such as defensive posture or analgesia encountered in fear reactions, associated with activation of the central nucleus of the amygdala[23]. Of the many brain areas to which the basolateral nucleus projects[24], the nucleus accumbens is the most likely to initiate a behavioural response to aversive environmental cues[25,26]. Interestingly, the RAIC has a direct projection to the nucleus accumbens[27,28], indicating that interactions between direct and indirect cortical afferents might occur there to modulate nociceptive responses.

Thus, the cerebral cortex modulates pain by acting on both pronociceptive and antinociceptive circuits. This dual effect is probably

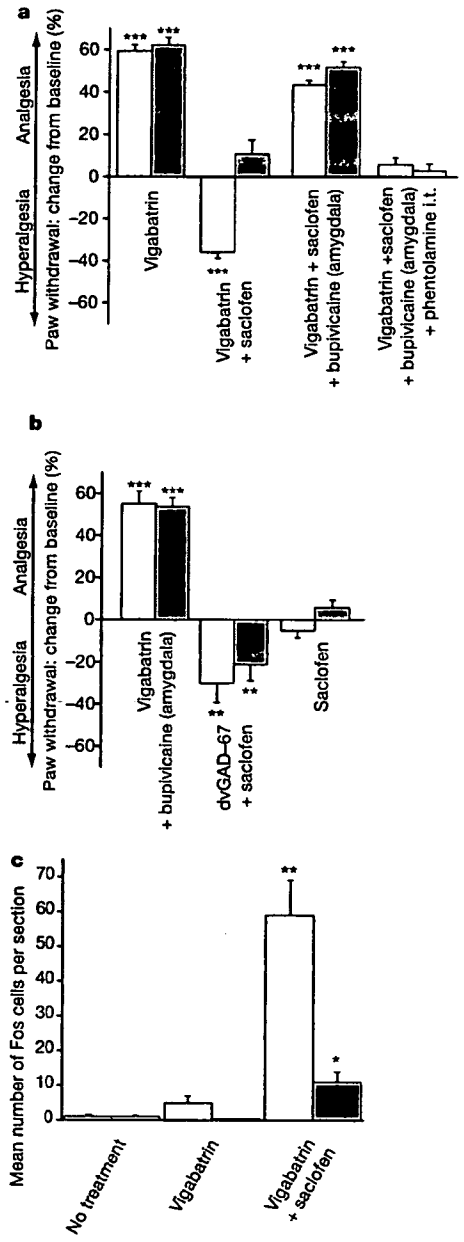

Figure 3 Nociceptive threshold and Fos immunoreactivity after drug treatment. **a**, Changes in nociceptive heat paw-withdrawal responses after the successive injection of four drugs. Each set of bars shows the behavioural response after the injection of the additional drug noted. Vigabatrin and saclofen were injected into the RAIC and the other drugs into the locations indicated. White bars, left paw; grey bars, right paw. **b**, Bupivicaine in the amygdala had no effect on the analgesia produced by vigabatrin alone in the RAIC. Hyperalgesia was also produced by injection of saclofen into the RAIC 2 days after injection of the GAD-67-expressing viral vector dvGAD-67. Injection of saclofen alone into the RAIC did not alter the nociceptive threshold. White bars, left paw; grey bars, right paw. **c**, Number of Fos-immunoreactive cells in the amygdala. White bars, basolateral nucleus; grey bars, central nucleus. One asterisk, $P < 0.05$; two asterisks, $P < 0.01$.

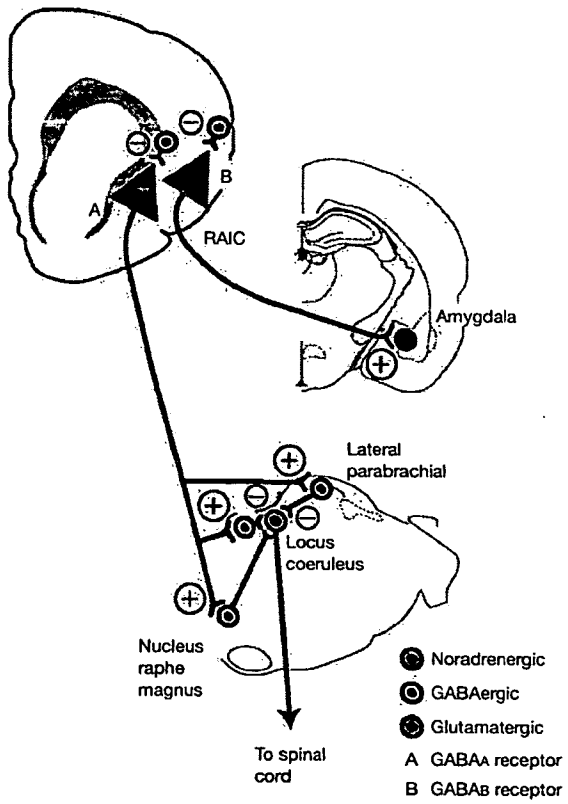

Figure 4 Summary of the main findings of the present study. From the RAIC, at least two independent projections modulate the nociceptive threshold, one to the amygdala and the other to the GABAergic neurons projecting to noradrenergic bulbo-spinal neurons. Both projections are modulated by local cortical GABAergic neurons. $GABA_A$ receptor stimulation (A) predominantly blocks the efferents to the peri-coerulear area, whereas $GABA_B$ receptor stimulation (B) blocks mainly the efferents to the amygdala. At least part of the projection from the RAIC to the peri-coerulear region terminates on GABAergic interneurons, which in turn are presumed to project to noradrenergic neurons in the locus coeruleus.

a defining feature of endogenous pain modulation[17,21], and we speculate that an imbalance in the cortical output is likely to underlie some chronic pain states. □

Methods

Experimental animals

We used 252 male Sprague–Dawley rats (270–320 g; Bantin-Kingman). Procedures for the maintenance and use of the experimental animals conformed to the regulations of the UCSF Committee on Animal Research.

Implantation of intracerebral cannulae

A stainless steel guide cannula (26-gauge, Plastics One) was cemented over a burrhole drilled over the RAIC (anterior-posterior (AP) 11.0, lateral (lat) 3.5 mm), and/or the amygdala (AP 6.20, lat 4.8 mm), and/or the locus coeruleus (AP −0.80, lat 1.2 mm), with interaural zero as the reference point. The guide cannula did not extend below the bone, to avoid any damage to the brain. Cortical injections were unilateral because stimulation of the RAIC with drugs was previously shown to produce bilateral effects[4]. On the day of testing (10 days after surgery), a 33-gauge bevelled injection cannula was inserted through the guide cannula to a distance 5.8, 8.0 or 6.0 mm below the cortical surface for the RAIC, amygdala or locus coeruleus, respectively. Drugs and viral vectors were injected over a 1-min period with a microinfusion pump.

Intracerebral injections of tracers

Fluoro-Gold (4%; 10–40 nl) in double-distilled water or 10% biotin–dextran (Molecular Probes) in PBS pH 7.4 was injected through a micropipette (40-μm tip). A period of 2–4 days was allowed for tracer transport.

Nociceptive testing

A treatment-blind researcher conducted the behavioural experiments. Hindpaw responses to radiant heat were measured with a commercial heat paw-withdrawal device (Plantar Analgesia Instrument; Ugo Basile, Comerio, Italy) in accordance with a standard protocol[15]

Open-field test

The open-field test for motor and behavioural impairment was performed in accordance with a standard protocol[15].

Histology

Aldehyde-fixed brains were cut transversely (40 μm slices) and kept in rostro-caudal order. Intracerebral drug injection sites were mapped on serial Nissl-stained sections. Histochemistry for β-galactosidase and acid phosphatase[29], and immunocytochemistry, were performed in accordance with standard protocols. Guinea-pig anti-$GABA_B$ antiserum was provided by Dr Margeta-Mitrovic (Departments of Physiology and Biochemistry, University of California, San Francisco)[30], and rabbit anti-fos antiserum was provided by Dr. D. Slamon (Department of Medicine, University of California, Los Angeles). Rabbit anti-GAD-67 (Ab5862) antiserum was purchased from Chemicon, and mouse monoclonal anti-GABAA (05-474) from Upstate Biotechnology.

Stereology and analysis of immunolabelled cells

Counts of cells per unit area of the cortex or amygdala were performed with an unbiased counting method with the use of a computer-aided system (Stereo Investigator; MicroBrightField).

Drugs

Vigabatrin, saclofen (Tocris Cookson), phentolamine (Sigma-Aldrich) and bupivicaine (AstraZeneca) were dissolved in 0.9% saline.

Viral vectors

Double-cassette-defective HSV vectors (dvGAD-67 and dvAP) were generated from amplicon plasmids, pHCL-CGAD-67 encoding rat GAD-67 (obtained from A. Tobin, University of California, Los Angeles) and *Escherichia coli* LacZ, and pHCL-CAP encoding *E. coli* LacZ and human acid phosphatase[29], with HSV-1 tsK as helper virus[8]. The titre of the defective vectors (in defective particle units) was determined by histochemistry with 5-bromo-4-chloro-3-indolyl-β-D-galactoside. *In vitro*, infection of cultured cerebellar granule cells (CGCs)—predominantly glutamatergic—or of cultured astrocytes resulted in both the expression of GAD-67 and the synthesis of GABA. GABA is released tonically from dvGAD-67-infected astrocytes and in a stimulus-evoked fashion from CGCs[8,9]. Expression from these vectors, with the CMV_{IE} promoter/enhancer, continues for about 7–10 days *in vivo*. Control rats were injected with a defective HSV vector expressing alkaline phosphatase and LacZ.

Statistical analysis

By using the outcome variable in each experiment, comparisons of treatment groups were made with Student's *t*-test or analysis of variance; $P < 0.05$ was considered statistically significant. Post-hoc analysis of means and s.e.m. with Scheffé's *F*-test confirmed statistical significance.

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that of mice lacking components of the hepatocyte growth factor/Met signaling pathway (*25*, *26*). In the adult organism, an analogous mechanism could explain the degenerative changes observed in heterozygote old mice. Although in young heterozygote *Dnchc1* mutants, the clinical manifestation of the developmental phenotype is not substantial, the age-related progressive neurodegeneration could be the consequence of a constantly reduced supply of trophic factors (such as NGF) caused by impaired dynein-dependent retrograde axonal transport.

We also identified a specific abnormality in the migration of facial motor neurons. A missense mutation in the p150^{Glued} subunit of dynactin has recently been identified in a human kindred displaying a SBMA-like syndrome dominated by facial, bulbar, and distal extremity weakness (*27*). This further supports the hypothesis that subtle alterations of the dynein/dynactin system can lead to human MND; mutations in anterograde axonal transport proteins, including the microtubule motor kinesin (KIF1B), can lead to slow progressive motor neuronopathy (*28*), indicating the potential generality of the link between retrograde and anterograde axonal motor protein deficits and motor neuron degeneration.

The *Loa* and *Cra1* mutations exhibit remarkable similarities to specific features of human pathology, including Lewy body–like inclusions containing SOD1, CDK5, NFs, and ubiquitin. Abnormal accumulation of NFs in the cell body and proximal axons is the most frequently seen early pathological characteristic of ALS (*29*). Retrograde transport of NFs is dynein-dependent, suggesting that the buildup of NFs is secondary to the defect in retrograde transport. SOD1 deposition, which may also be dynein-dependent (*4*), is an unexpected finding pointing to a link between dynein dysfunction and normal SOD1 function.

The mechanism by which SOD1 mutations bring about motor neuron degeneration is unknown. Recent data have demonstrated the slowing of axonal transport as an early event in the motor neuron toxicity of SOD1 mutants (*30*). The disruption of dynein function by interaction with mutant SOD1, creating an aberrant protein complex that interferes with the function of the wild-type complex, may also provide a link between axonal transport, NF accumulation, and cell death.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/300/5620/808/DC1
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GABA and Its Agonists Improved Visual Cortical Function in Senescent Monkeys

Audie G. Leventhal,[1]* Yongchang Wang,[1] Mingliang Pu,[1] Yifeng Zhou,[2] Yuanye Ma[3]

Human cerebral cortical function degrades during old age. Much of this change may result from a degradation of intracortical inhibition during senescence. We used multibarreled microelectrodes to study the effects of electrophoretic application of γ-aminobutyric acid (GABA), the GABA type a (GABAa) receptor agonist muscimol, and the GABAa receptor antagonist bicuculline, respectively, on the properties of individual V1 cells in old monkeys. Bicuculline exerted a much weaker effect on neuronal responses in old than in young animals, confirming a degradation of GABA-mediated inhibition. On the other hand, the administration of GABA and muscimol resulted in improved visual function. Many treated cells in area V1 of old animals displayed responses typical of young cells. The present results have important implications for the treatment of the sensory, motor, and cognitive declines that accompany old age.

Aging is known to adversely affect visual function in humans. Senescent humans exhibit decreased visual acuity, binocular summation, contrast sensitivity, motion sensitivity, and wavelength sensitivity. The elderly also respond much more slowly in visual tests and do not perform as well at shape discrimination tests as do the young and middle aged (*1*–*10*). It has been hypothesized that many of the foregoing declines during old age are due to degeneration and/or dysfunction in central visual areas (*10*, *11*).

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The receptive field properties of cells in the visual cortex (area V1) have been studied for over 40 years. The cells in area V1 are known to respond selectively to both the angular orientation and the direction of motion of lines, bars, and edges (*12*). The orientation- and direction-selective responses of V1 cells are thought to participate in the perception of form and motion.

We reported previously (*11*) that primate visual cortical function declines because V1 cells in old (26 to 30 years old) macaque monkeys exhibit decreased orientation and direction selectivity, accompanied by increased visual responsiveness, increased spontaneous activity, and a decreased ability to signal visual stimuli above background activity (signal-to-noise ratio). We have now studied the effects of electrophoretic application of the inhibitory transmitter γ-aminobutyric acid (GABA), the GABA type a (GABAa) agonist muscimol, and

the GABAa antagonist bicuculline on the receptive field properties of individual V1 cells in old monkeys. We tested the hypothesis that the application of GABA and GABA agonists on individual V1 cells can improve visual function in old animals.

We studied a total of 242 neurons in six young monkeys (7 to 9 years old) and 257 neurons in seven old monkeys (26 to 32 years old) (fig. S1). Both *Macaca mulatta* (75% of cells) and *M. fascicularis* (25% of cells) were studied. The results for the two species did not differ and thus are combined in the figures. Some of the animals included in this study also provided data for a previous one (*11*).

The effects of GABA, muscimol, and bicuculline on the responses of two typical cells in old monkeys and one in a young monkey are illustrated in Fig. 1. Before drug administration, cells in old animals responded equally well to all orientations and directions (Fig. 1, A and D). After GABA and muscimol administration, some of these cells

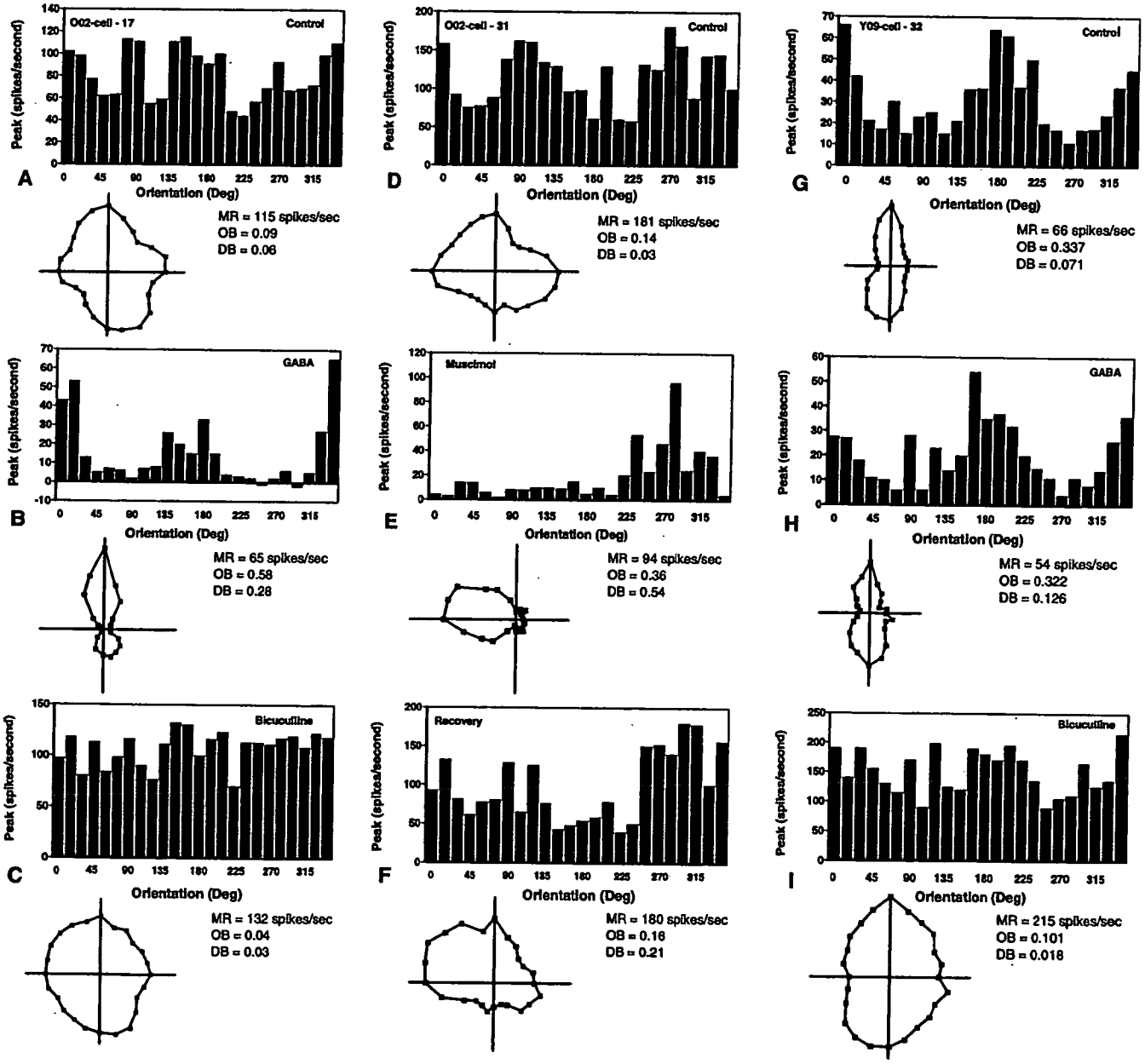

Fig. 1. Tuning curves and corresponding polar plots obtained for two representative cells in old monkeys (A to F) and one typical cell from a young monkey (G to I) that received treatment with GABA, muscimol, and bicuculline. The maximum (peak) responses (MR), orientation biases (OB), and direction biases (DB) are shown for each condition. A typical old cortical cell showing a lack of orientation and direction sensitivity is shown in (A). Three minutes after GABA application (B), this cell exhibited strong orientation and moderate direction selectivity. The cell's peak response decreased, as did its spontaneous activity. GABA application was then discontinued, and bicuculline application was begun (C). Bicuculline reversed the effects of GABA. The responses of a second cell in visual cortex of an old monkey showing a degradation of orientation and direction selectivity are shown in (D). Three minutes after muscimol administration (E), this cell exhibited moderate orientation selectivity, very strong direction selectivity, a decreased peak response, and decreased spontaneous activity. Five minutes after the discontinuation of muscimol administration, the drug-induced improvement disappeared (F). A tuning curve typical of the majority of cells in young monkeys is shown in (G). The cell was selective, and its selectivity was not affected by the administration of GABA (H). On the other hand, application of bicuculline resulted in a more than 300% increase in peak response and greatly reduced orientation selectivity (I).

responded strongly to a narrow range of preferred orientations and directions and exhibited nearly no response to the nonpreferred orientations and directions (Fig. 1, B and E). This differential effect on preferred and nonpreferred orientations and directions suggests that the effect of GABA is not simply to nonselectively decrease the responses of the cell to all visual stimuli. Additionally, as illustrated in Fig. 1H, GABA administration did not affect the selectivity of most of the already strongly selective cells (Fig. 1G) in young animals.

We also investigated the effects of the application of bicuculline on cells in young and old animals. In the old animals, bicuculline did not change stimulus selectivity to any great extent and resulted in a somewhat increased visual response (Fig. 1C). In contrast, as has been reported previously (*13*, *14*), bicuculline greatly diminished selectivity in young animals and greatly increased the magnitude of the visual response (Fig. 1I).

The effects of GABA and muscimol administration on the orientation and direction selectivities of all V1 cells studied in old monkeys are summarized in Table 1. Both GABA and muscimol resulted in increases in the percentage of cells that exhibited significant orientation and direction selectivity. In fact, the percentages of orientation- and direction-selective cells approached those seen in normal animals after the application of GABA and muscimol. On average, however, the degree of selectivity of most cells in old monkeys after drug administration was still lower than that seen in young animals (Table 1, legend). Additionally, unlike in young animals in which bicuculline greatly diminishes selectivity (*13*, *14*), bicuculline did not significantly affect the already low percentage of direction-selective cells in old animals. The number of orientation-selective cells was only reduced slightly (Table 1).

The effects of drug administration on the visually evoked response and spontaneous activity of V1 cells in old monkeys are summarized in Fig. 2, A and B, respectively. GABA and muscimol both decreased peak visual response and spontaneous activity (t test, $P < 0.01$ in each case). Muscimol was more potent than GABA, and its application resulted in responses that were within the normal range. Bicuculline had the opposite effect and resulted in increased visual and spontaneous responses (t test, $P < 0.001$ in both cases). In each case, 5 to 10 minutes after the cessation of drug administration, the cells reverted to the preapplication state.

Proper brain function requires that stimuli evoke reliable responses that are easily discernable from background activity. We reported previously (*11*) that the ratio of the visually evoked response to background activity was much lower in old than in young monkeys. Histograms showing effects of drug application on the ratio of the peak visually evoked response to the spontaneous discharge rate (referred to here as signal-to-noise ratio) of cortical cells are presented in Fig. 2C. Both GABA and muscimol administration resulted in higher ratios (t test, $P < 0.01$ in both cases) and thus an improved ability to signal visual stimuli. On the other hand, bicuculline did not affect signal-to-noise ratios significantly in old animals because ratios were already low.

If GABA-mediated inhibition degrades during old age, then GABA and GABA agonists should be more effective in old than in young animals. Conversely, GABA antagonists should be more effective in young than in old monkeys. The results in Table 2 show that GABA and muscimol both result in larger percentage decreases in visually

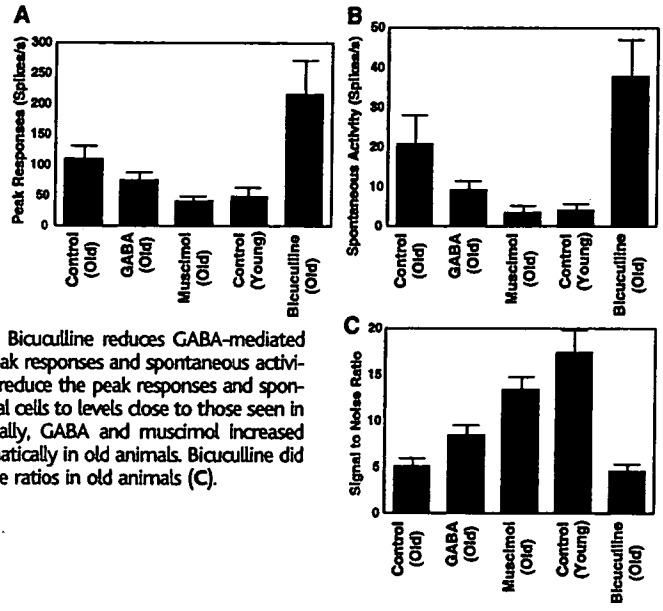

Fig. 2. The maximum visually evoked responses (A) and spontaneous activities (B) of V1 cortical cells in untreated old monkeys, untreated young monkeys, and old monkeys treated with GABA, muscimol, and bicuculline. Cortical cells in old monkeys exhibit abnormally high peak responses and spontaneous activities as compared to young monkeys. Bicuculline reduces GABA-mediated inhibition and increases peak responses and spontaneous activities. GABA and muscimol reduce the peak responses and spontaneous activities of cortical cells to levels close to those seen in young monkeys. Additionally, GABA and muscimol increased signal-to-noise ratios dramatically in old animals. Bicuculline did not improve signal-to-noise ratios in old animals (C).

Table 1. Effects of drug application on the percentages of orientation- and direction-selective cells in area V1 of old monkeys. Old monkeys exhibit a reduction in orientation- and direction-selective cells as compared to young monkeys. Bicuculline results in a small decrease in the number of orientation-selective cells and no change in the number of direction-selective cells. In contrast, GABA and muscimol are capable of increasing the orientation- and direction-selective responses of cortical cells. Although many cells in old monkeys do become selective after drug application, most still do not exhibit the strong selectivities seen in young animals. In old animals, the mean orientation bias increased from 0.098 ± 0.031 to 0.148 ± 0.053 (mean ± SD), whereas the mean direction bias increased from 0.061 ± 0.022 to 0.114 ± 0.035 (mean ± SD) after GABA application. These values are lower than the average biases seen in young animals. Our results for young animals indicate mean biases of 0.37 for orientation and 0.2 for direction (*11*).

	Old monkey				Young monkey control
	Control	GABA	Muscimol	Bicuculline	
Orientation selective	39%	81%	73%	22%	88%
Direction selective	23%	63%	68%	24%	69%

Table 2. Effects of drug application on responses of V1 cells. Changes in peak visually evoked response and spontaneous firing rate induced by GABA, muscimol, and bicuculline in young and old monkeys. GABA and muscimol resulted in larger percentage decreases in neuronal firing rates in old than in young monkeys. Bicuculline resulted in larger percentage increases in neuronal firing rates in young than in old monkeys.

		Control	GABA	Muscimol	Bicuculline
Old monkeys	Peak response	100%	−28.8%	−61.1%	+40.4%
	Spontaneous rate	100%	−57.6%	−83.4%	+88.4%
Young monkeys	Peak response	100%	−20.1%	−28.7%	+214.2%
	Spontaneous rate	100%	−11.2%	−9.1%	+416%

evoked and spontaneous activity in old than in young animals (t test, $P < 0.01$ in both cases). Bicuculline, on the other hand, resulted in larger percentage increases in visually evoked and spontaneous activity in young monkeys than in old ones (t test, $P < 0.01$). Taken together these results strongly suggest a decrease in the amount of GABA-mediated inhibition in cortex.

The results of this study show that the administration of GABA and muscimol results in improved orientation and direction selectivity, accompanied by decreased visual responsiveness, decreased spontaneous activity, and an increased ability to signal visual stimuli. Some cells in V1 of old animals displayed responses typical of cells in young animals after drug application. A restoration of function was evident as soon as 2 minutes after drug delivery. Five to 10 minutes after discontinuation of drug administration, neuronal function reverted to preapplication levels. Application of bicuculline resulted in much smaller changes in the properties of old V1 cells than in the properties of young ones. It should be mentioned that the iontophoretic application of noradrenaline is reported to improve the signal-to-noise ratio of cells in cat visual cortex (*15*) and primate motor cortex (*16*). Whether age affects noradrenaline levels in primate cortex is not known.

The foregoing results are consistent with the hypothesis that reductions in GABA-mediated intracortical inhibition contribute to the degradation of cortical function that accompanies old age. Our finding that GABA agonists exert a weaker effect on cortical cells in young monkeys than in old ones further supports this idea. The finding that bicuculline is more effective in young than old monkeys is also compatible with this suggestion.

A decrease in intracortical inhibition could result from diminished release of transmitter, diminished production of transmitter, a degradation of transmitter receptors, membrane changes, etc. Although our findings cannot pinpoint the changes in old monkeys, the present findings show that simply adding GABA and GABA agonists does facilitate visual function in old animals. Thus, it is tempting to speculate that normal aging results in a decreased ability to produce GABA in cerebral cortex. It is noteworthy that an age-related degradation of GABA-mediated inhibition has also been reported in the inferior colliculus. The effects of age on the inferior colliculus include reductions in the number of GABA-immunoreactive neurons, the concentration of GABA, GABA release, and GABA receptor binding (*17*, *18*). Similar studies in primate cortex need to be carried out.

Some V1 cells in old animals exhibited responses typical of cells in young ones after the application of GABA and GABA agonists. However, most cells exhibited only partial recovery as a result of drug administration. Thus, factors other than a degradation of GABA-mediated inhibition may also be involved. Because GABA-mediated inhibition is prevalent throughout the neocortex (*19*, *20*), it is likely that changes similar to those seen in V1 will exist in many cortical areas in old animals. Thus, the improvement in function of V1 cells after the application of GABA and its agonists has important implications for the treatment of the sensory, motor, and cognitive declines that accompany old age.

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Supporting Online Material

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Materials and Methods
Fig. S1

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